MONASH University

Programmed Cell Death in *Legionella* Infection

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BSc Hons (I) Biochemistry

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Abstract

Legionella ssp. are the causative agents of severe inflammatory pneumonia, known as Legionnaires' Disease, which is fatal in up to 30 % of cases. *Legionella* replicate within alveolar macrophages by hijacking host cell pathways to establish a unique vacuolar niche. This includes the regulation of programmed host cell death factors, as *Legionella* must first prevent, and then induce, host cell death to promote bacterial growth and egress, respectively. However, the molecular mechanisms involved in toggling "off" and "on" host cell death signalling pathways remain undetermined. The major focus of the work described in this thesis was the delineation of the role that programmed host cell death pathways play in *Legionella* infection. To do this, a novel live-cell imaging technique was employed to visualise the intracellular life-cycle of *Legionella* and to monitor macrophage health in real-time. Using this method, I was able to confirm that wild-type *Legionella* induce a rapid form of cell death, termed pyroptosis, which is dependent on bacterial flagellin and the host protease, caspase-1. While flagellin/caspase-1-mediated pyroptosis prevents bacterial replication, I have identified that aflagellated *Legionella* also induce caspase-11-dependent pyroptosis. In contrast to caspase-1, caspase-11-mediated pyroptosis is induced in the late stages of infection, concomitant with *Legionella* egress, and does not interfere with intracellular bacterial replication. *Legionella* are also thought to induce other forms of host cell death, however, genetic ablation of mitochondrial apoptosis (BAX and BAK deletion), caspase-independent necroptotic cell death (RIPK3 and MLKL deletion), or BNIP3 and BCL-RAMBO, the putative targets of the effector protein SidF, did not affect *Legionella* replication or the killing of host macrophages.

Legionella must prevent the activation of host cell death signalling to allow for efficient replication. While down-regulation of flagellin enables intracellular growth in the presence of caspase-1, little is known about how *Legionella* might evade apoptotic cell death. Using live-cell imaging, I have now shown that *Legionella*-infected macrophages depend critically upon the anti-apoptotic activity of host cell BCL-XL, but not other BCL-2 family members, for viability. In the absence of BCL-XL, *Legionella*-infected cells underwent apoptosis, which abolished bacterial replication and dissemination. *Legionella* infection could be fully restored by inhibiting mitochondrial apoptosis, either via BAX/BAK deletion or caspase inhibition. A single dose of BCL-XL-targeted BH3-mimetic therapy significantly reduced *Legionella* burden in the lungs of mice and prevented lethal bacterial infection. Mechanistically, I identified that *Legionella* infection inhibits host protein synthesis, which sensitises macrophages to BCL-XL loss or inhibition, via depletion of another anti-apoptotic BCL-2 family protein, MCL-1. Together, these results demonstrate that *Legionella*-infected macrophages are specifically and acutely sensitive to apoptotic cell death following the loss, or inhibition, of BCL-XL. Thus, the re-purposing of existing drugs, such as chemotherapeutic BH3-mimetics, to target host, rather than bacterial, pathways represents a novel and promising strategy for the treatment of intracellular pathogens that show increased, and often rapidly acquired, antibiotic resistance.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Thesis including published works General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal and one unpublished publication. The core theme of the thesis is the role of host cell death pathways during *Legionella* infection. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Biochemistry and Molecular Biology Department under the supervision of Dr Thomas Naderer and Dr James Vince.

In the case of Chapters One and Four, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent (%) of students contribution
1	Programmed Cell Death in *Legionella* infection	Published	80 %
4	Genetic or pharmacological inhibition of BCL-XL controls *Legionella* infection by inducing apoptosis of infected macrophages	Returned for revision	Experimental work: 95 % Writing: 75 %

I have not re-numbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main supervisor signature: [redacted] **Date:** 16/11/2015

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Acknowledgments

First and foremost, I would like to thank my supervisors Dr Thomas Naderer and Dr James Vince for all their support, guidance, and encouragement over the past four years. I feel incredibly grateful to have been a part of such a supportive, capable, and innovative team, and I appreciate all of the freedom and opportunities you have provided to help me grow and learn as a scientist.

To all members, past and present, of the Lithgow, Traven and Naderer labs for sharing your ideas, advice, and reagents, and for helping out with experiments when needed. Most especially, thanks to Gilu, who was always willing to lend an extra pair of hands and who was, I am sure, the only person standing between our lab and utter chaos.

Thanks to the Hartland and Vance Labs for lending us the *Legionella* strains we needed, and to David Huang, Guillaume Lessene, Lorraine O'Reilly, Seth Masters, David Segal, Stefan Glasser, Philippe Bouillet, and Andreas Strasser at WEHI who supplied the mice, macrophages, reagents, and antibodies etc. that made this project possible. Especial thanks to members of the Vince Lab, including James and Dr Kate Lawlor, for your work towards some of the experiments in Chapters 4 and 5. Thank you to the technical staff at Monash Micro Imaging for your assistance, and to Dr Keith Schulze for writing the code we needed to run our live-cell imaging analyses.

Thank you to Victoria Hewitt for the advice about putting presentations together, teaching undergraduate students, and dealing with supervisors. Also, for teaching me to play Agricola in-between experiments on Saturday afternoons. Thanks to Dan Newman for all your help with the statistics in this thesis, and to Sam Palframan for fixing my computer on more than one occasion.

Thank you to my friend Claudette Kotze for reminding me that there is life outside the lab, and for dragging me out into it once in a while. I appreciated it, even if I didn't always let on at the time.

Finally, to my parents; thanks for all your support, love, and encouragement over the years. From that first middle-of-the-night phone call to Germany, to endlessly reassuring me in the face of failures, to proof-reading my entire thesis in all its many iterations, you have never wavered in your support of me and I could not have done any of this without you. Also, where would I be without all the doggy hugs I have gotten over the years, so thanks to Megan and Freya and, of course, to Jasper for the nibbles.

Preface

The contributions of collaborators are outlined below:

i. Dr Kate Lawlor (WEHI) performed the IL-1β and TNF quantification by ELISA on the lung lysates harvested from the *Bcl-x*$^{flox/flox}$*;LysM-Cre* and *Bcl-x*$^{flox/flox}$ mice (Chapter 4, Fig. 4j).

ii. Dr Kate Lawlor performed the phenotyping of the *Bcl-x*$^{flox/flox}$*;LysM-Cre* mice (Chapter 4, Extended data Fig. 4c and d).

iii. Dr James Vince (WEHI) treated the BMDMs derived from the *Bcl-x*$^{flox/flox}$*;LysM-Cre* and *Bcl-x*$^{flox/flox}$ mice with translation inhibitors (Chapter 5, Fig. 5.7a and b).

iv. Dr Thomas Naderer (Monash) performed the FACS analysis on the WT and MCL-1-fN BMDMs treated with translation inhibitors and ExoA, or infected with *Legionella* (Chapter 5, Fig. 5.5 and 5.6).

v. Dr Adam Vogrin (Monash) performed the live-cell imaging of BMDMs infected with the Δ*icmS* mutant (Chapter 5, Fig. 5.9).

vi. Seong Chow (Monash) performed the ^{35}S methionine-labelling experiments (Chapter 5, Fig. 5.8).

All other experiments comprise my own work.

Publications and Presentations

Manuscripts Arising From This Thesis

Refereed Journals

Speir, M., Glaser, S. P., Schulze, K. E., Schuelein, R., O'Reilly, L. A., Mason K., Hartland E. L., Lithgow, T., Strasser, A., Lessene, G., Huang, D. C. S., Vince, J. E. and Naderer, T. (2015) Genetic or pharmacological targeting of BCL-XL controls *Legionella* by inducing apoptosis of infected macrophages. Submitted to Journal of Experimental Medicine (J.E.M).

Reviews

Speir, M., Vince, J. E. and Naderer, T. (2014) Programmed cell death in *Legionella* infection. *Future Microbiology*. **9**: 107-118.

Abstracts Arising From This Thesis

Oral Presentations

Speir, M., Vince, J. E., Naderer, T. (2015) Targeting of BCL-XL controls *Legionella* infection by inducing apoptosis of infected macrophages. Molecular Analysis of Bacterial Pathogens (BacPath), Philip Island, VIC, Australia (Sept 27^{th} – 30^{th}).

Speir, M., Vince, J. E., Naderer, T. (2014) Anti-apoptotic BCL-XL is the linchpin of *Legionella* infection. Monash University Microbiology Student Conference, Melbourne, VIC, Australia (Jul 16^{th} – 17^{th}).

Poster Presentations

Speir, M., Vince, J. E., Naderer, T. (2015) Genetic or pharmacological targeting of BCL-XL controls *Legionella* infection by inducing apoptosis of infected macrophages. Lorne Infection and Immunity, Lorne, VIC, Australia (Feb 18^{th} – 20^{th}).

Speir, M., Vince, J. E., Naderer, T. (2013) Manipulation of host cell death pathways by *Legionella pneumophila*. International Conference on *Legionella*, Melbourne, VIC, Australia (Oct 29^{th} – Nov 1^{st}).

Speir, M., Vince, J. E., Naderer, T. (2013) Manipulation of host cell death processes by *Legionella pneumophila.* Microbiology@Monash Plenary Meeting, Melbourne, Victoria, Australia (Jun 6^{th}).

List of Abbreviations

AIM2	Absent in melanoma 2
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
Bcl	B-cell lymphoma
BCL-RAMBO	Bcl-2-like *protein* 13
BCYE	Buffered charcoal yeast extract
BH	Bcl-2 homology (domain)
BID	BH3 interacting-domain death agonist
BIM	Bcl-2-like *protein* 11
BMDM	Bone marrow-derived macrophage
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
C-terminal	Carboxyl-terminal
CARD	Caspase activation and recruitment domain
Caspase	Cysteine-dependent aspartic acid-specific protease
CFU	Colony forming unit
CTG	Cell tracker green
DISC	Death-induced signalling complex
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
DISC	Death-induced signalling complex
FACS	Fluorescence-activated cell sorting
FasL	Fas-associated ligand
FBS	Foetal bovine serum
GFP	Green fluorescent protein
h	Hours
HCT	Haematocrit
HGB	Haemoglobin
IAP	Inhibitor of apoptosis proteins
IMS	Inter-membrane space
IPAF	see NLRC4
LAMP-1	Lysosomal-associated membrane protein 1
LCV	*Legionella*-containing vacuole
MAPK	Mitogen-activated protein kinase
MCL-1	Induced myeloid leukaemia cell differentiation *protein*-1
MEK	Mitogen-activated protein *kinase kinase* (MAPK2)
MLKL	Mixed lineage kinase domain-like
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilisation

NF-κB	Nuclear factor-kappa B
NAIP	NLR family apoptosis inhibitory protein
NDB-LRR	Nucleotide binding domain-leucine-rich repeat
NLR	NOD-like receptor
NLRC4	NLR family CARD domain-containing *protein* 4
NOD	Nucleotide oligomerisation domain
NOXA	Phorbol-12-myristate-13-acetate-induced *protein* 1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PUMA	p53 up-regulated modulator of apoptosis *protein*
Q-VD-OPh	(3S)-5-(2,6-Difluorophenoxy)-3-[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate
RBC	Red blood cell
RIPK	Receptor-interacting protein kinase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
TM	Trans-membrane
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
WT	Wild-type

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Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Writing/Editing	80 %

The following co-authors contributed to the work:

Name	**Nature of contribution**
Thomas Naderer	Writing/Editing
James Vince	Writing/Editing

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature	[redacted]	**Date** 16/11/2105

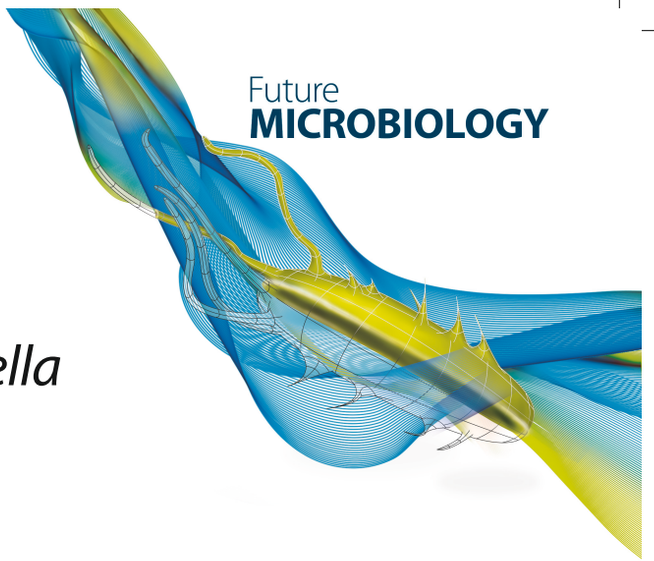
CHAPTER 1

PROGRAMMED CELL DEATH IN *LEGIONELLA* INFECTION

Chapter published as a Review:

Speir, M., Vince, J. E. and Naderer, T. (2014) Programmed cell death in *Legionella* infection. *Future Microbiology*. **9**: 107-118.

REVIEW



Programmed cell death in *Legionella* infection

Mary Speir[1], James E Vince[2,3] & Thomas Naderer*[1]

ABSTRACT: The causative agent of Legionnaires' disease, *Legionella pneumophila*, resides within alveolar macrophages by exporting 295 bacterial virulence proteins (effectors) to modulate host cell processes. This leads to the formation of a unique vacuolar niche and the suppression of macrophage cell death pathways, which, in turn, promote bacterial survival and allow sufficient time for replication. However, once nutrients within the vacuole are depleted, *Legionella* must act to induce host cell death in order to facilitate bacterial egress and reinfect new cells. Intracellular *Legionella* also evade detection by the host cell's innate immune system, which seeks to destroy invading pathogens by activating inflammasome complexes, thereby promoting proinflammatory cytokine activation and pyroptotic cell death. Understanding how different forms of programmed cell death contribute to *Legionella* infectivity and are manipulated by *Legionella* effector proteins will be important for identifying novel antibacterial therapeutic targets.

KEYWORDS
• bacterial pathogen
• caspase • inflammasome
• innate immunity
• macrophage

Cell death-induced innate immunity

Macrophages are key effectors of the innate immune system and are able to coordinate several antimicrobial immune responses. One of the most striking of these is the initiation of cellular suicide to promote killing of intracellular pathogens at the expense of the host cell [1]. It is becoming increasingly evident that several cell death pathways lie at the forefront of the innate immune response to invading pathogens [2,3]. For example, detection of specific microbial ligands can lead to inflammasome-mediated activation of the cysteine-aspartic protease, caspase-1, which promotes secretion of the proinflammatory cytokines IL-1β and IL-18, and induces an inflammatory form of cell death, termed pyroptosis [4]. Caspase-1-dependent cell death involves the rapid release of cytosolic contents, including bacteria, which are then targeted by activated neutrophils [3]. By contrast, classical mitochondrial apoptosis is driven by a loss of mitochondrial membrane integrity that culminates in the activation of caspase-3 and -7, and the ordered, noninflammatory dismantling of the cell [5]. Activation of apoptosis is thought to reduce bacterial load by blocking the cell cycle of several intracellular pathogens [1]. In addition, some microbes, such as a virulent *Mycobacterium tuberculosis*, become trapped inside apoptotic remnants and are delivered to hydrolytic phagolysosomes upon uptake by surrounding macrophages [6]. Although virulent *M. tuberculosis* inhibits apoptosis, it induces necrosis for efficient release and reinfection of bystander macrophages [6]. Finally, upon activation of specific TNF or Toll-like receptor (TLR) family members, programmed macrophage necrosis, known as necroptosis, can be triggered via the receptor-interacting protein kinases (RIP1 and RIP3) [2]. While necroptosis can protect against viral infection,

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possibly by removing pathogen-infected cells or activating inflammatory cytokines, a recent study has suggested that some bacteria may promote macrophage necroptosis to evade immune responses [7]. Thus, *Legionella* and other intracellular pathogens that reside long term within macrophages have evolved novel and diverse strategies to escape host surveillance mechanisms and to manipulate cell death signaling pathways to their own advantage.

• *Legionella pneumophila*

Legionella spp., including the most prevalent species *L. pneumophila*, are Gram-negative, aerobic bacteria that replicate preferentially within unicellular protozoan hosts. They are also opportunistic human pathogens and infection can result in two distinct clinical and epidemiological syndromes, Legionnaires' disease and Pontiac fever, known collectively as legionellosis [8]. Legionnaires' disease is a severe inflammatory pneumonia that primarily affects elderly and immunocompromised patients, while the milder respiratory illness Pontiac fever predominates in otherwise healthy individuals and is generally cleared by the host immune system [8].

Legionella infection follows the inhalation of aerosols derived from water sources, such as air conditioners and hot water distribution systems that are contaminated with *Amoebae* harboring the bacteria [8]. Within the lungs, *Legionella* are phagocytosed by resident alveolar macrophages [8] and, in mice, by recruited dendritic cells [9]. Macrophages are specialized phagocytes capable of engulfing whole cells destined for destruction within hydrolytic lysosomes, called phagolysosomes. Not surprisingly, most intracellular pathogens have evolved diverse mechanisms to either escape or stall phagocytic trafficking pathways to prevent phagosome maturation [10]. Only a few pathogens, including the Gram-negative bacteria *Coxiella* (etiological agent of Q-fever) and the protozoan parasite *Leishmania*, are known to replicate in mature phagolysosomes [11]. By contrast, phagosomes containing *Legionella* evade endocytic maturation and, instead, fuse with specific endoplasmic reticulum (ER)-derived vesicles to create a specialized organelle, termed the *Legionella*-containing vacuole (LCV), which provides a replicative niche (reviewed in [8,12]).

The dot/icm type IV secretion system

The formation and maintenance of the LCV, as well as continued bacterial replication, are dependent upon a functional type IV secretion system (T4SS). The T4SS exports effector proteins directly into the cytoplasm of the host cell. These effectors then interfere with multiple host cell processes, including trafficking and programmed cell death pathways **(Figure 1)** [8]. The T4SS is a macromolecular transport complex assembled from the products of 27 genes found at two loci, *icm* and *dot* [13,14]. Remarkably, the export of at least 295 *Legionella* effector proteins has been confirmed experimentally [15]. This number is close to 10% of the predicted total protein-encoding genome of *Legionella* [16,17]. *Legionella* effector proteins are structurally diverse and share little homology with other bacterial proteins in current databases, hampering protein function analysis [18]. Furthermore, genetic deletion of most effectors has little consequence for intracellular survival of *Legionella* [19]. Notably, however, a number of effectors show a high degree of similarity to eukaryotic protein sequences, supporting the concept of molecular mimicry [8]. In addition, there is considerable functional redundancy amongst *Legionella* effectors without conservation of specific protein sequences or enzymatic activities [10]. For instance, *Legionella* use several secreted effector proteins to post-translationally modify and regulate the activity of Rab1 (a small GTPase that controls trafficking between the ER and the Golgi) over the course of the infection (reviewed in [10]). It is likely that other trafficking pathways, including post-Golgi and autophagic vesicles, may be targeted by similarly complex interactions [20,21].

The innate immune response against *Legionella*

• Inflammasomes

Inflammasomes are large multiprotein complexes that can respond to the cytosolic presence of bacterial pathogen-associated molecular patterns (PAMPs) to promote caspase-1-dependent activation of IL-1β and IL-18, and a unique inflammatory form of cell death known as pyroptosis [22]. Microbial PAMPs are sensed by several nucleotide binding domain-leucine-rich repeat NOD-like receptors (NLRs), including NLRP3, NLRC4 and the HIN200 family member AIM2, to initiate inflammasome formation [4]. Genetic deletion of different inflammasome components has demonstrated their importance to host protection from bacterial, viral and fungal pathogens [4]. In addition to cytokine activation, recent studies

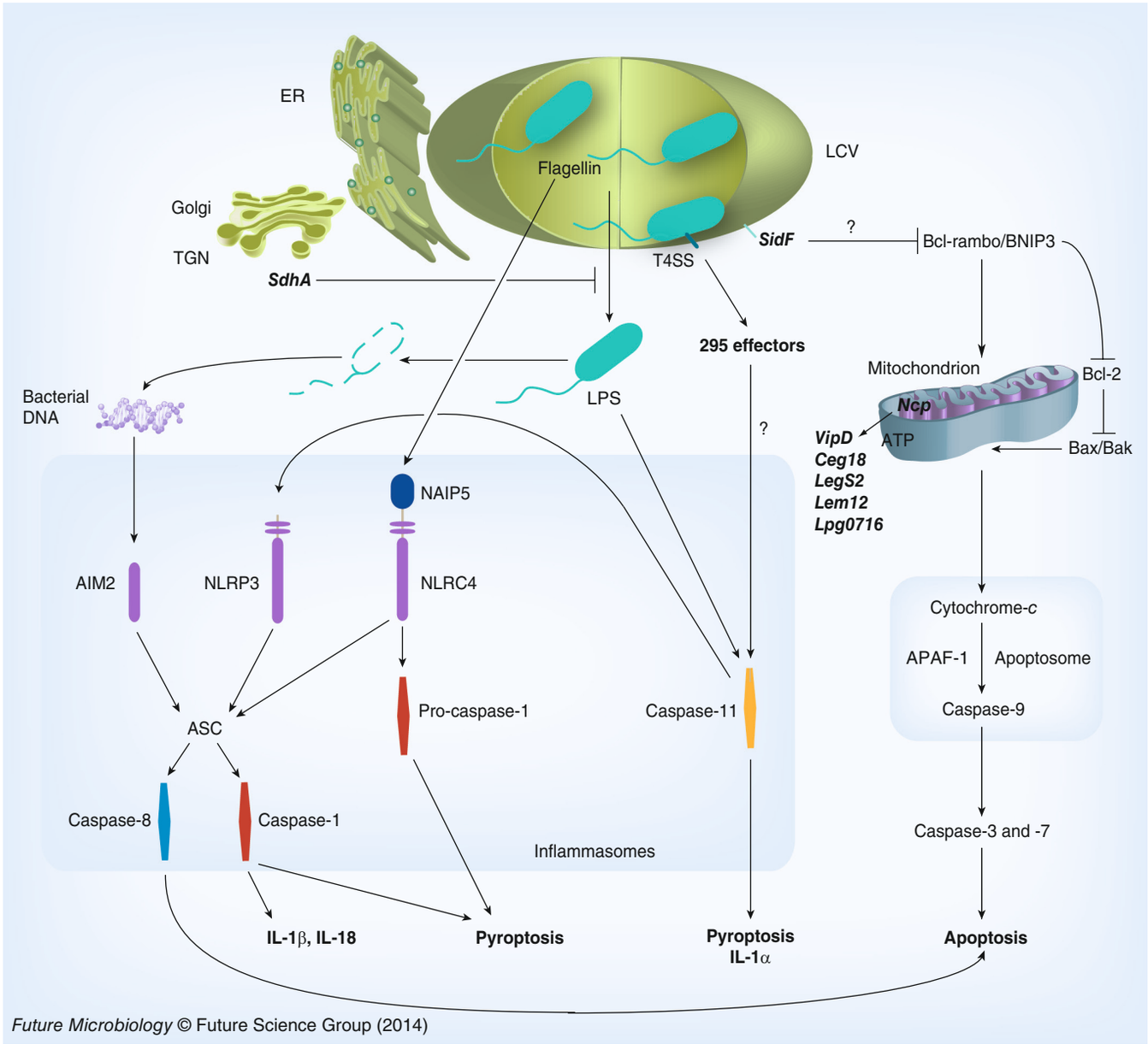

Figure 1. Programmed cell death signals targeted by *Legionella* effectors. *Legionella* replicate in special niches, termed LCVs, and export 295 effector proteins via their T4SS. While not an effector, flagellin is also thought to reach the cytosol via the T4SS, or by disruption of the LCV membrane, as observed in the *ΔsdhA* mutant. Flagellin is recognized by NAIP5 and activates the NRLC4/caspase-1 inflammasome. In addition, bacterial DNA is sensed by AIM2 to induce caspase-1-dependent pyroptosis. Cell death is also induced via a noncanonical inflammasome depending upon LPS and caspase-11, which can also modulate the NRLP3 inflammasome. Inflammasome complex formation depends upon homotypic protein–protein interactions via pyrin or card domains. Finally, several effectors target mitochondria to either inhibit or induce apoptosis, which is regulated via BH3-containing prodeath factors, BNIP3 and Bcl-rambo, antideath Bcl-2, and Bax/Bak. Release of cytochrome c induces apoptosome formation, mediated by the adapter protein Apaf-1, and activation of the executioner caspase-3 and -7.
ER: Endoplasmic reticulum; LCV: *Legionella*-containing vacuoles; LPS: Lipopolysaccharide; T4SS: Type IV secretion system; TGN: Trans-Golgi network.

demonstrate that inflammasome-mediated pyroptosis and inflammatory eicosanoid release may also play a role in bacterial clearance [23]. In mice, pyrin domain-containing inflammasomes, such as NLRP3 and AIM2, recruit and activate caspase-1 indirectly via the adaptor protein ASC

(Figure 1). NLRC4 also requires ASC to efficiently recruit and activate caspase-1 in order to generate mature IL-1β, but may interact directly with caspase-1 via CARD–CARD homotypic interactions to cause cell death **(Figure 1)**.

Pyroptosis follows inflammasome formation and activation of caspase-1 or caspase-11 (see 'NLRC4 inflammasome' and 'Caspase-11 inflammasome' sections), and occurs in the absence of apoptotic caspase function [3]. However, there is cross-talk between pyroptotic and apoptotic pathways, as recent studies demonstrate that NLRP3 or AIM2 inflammasome formation can result in ASC-dependent recruitment and activation of apoptotic caspase-8 [24,25]. Similarly, caspase-8 can directly cleave precursor IL-1β at the same cleavage site used by caspase-1 to induce IL-1β activation and secretion [26,27]. However, caspase-8 activation does not always appear to result in IL-1β cleavage and how the cell decides between caspase-8-driven apoptosis and IL-1β cleavage remains to be elucidated [25].

• NLRC4 inflammasome

Bacterial flagellin, the major protein component of prokaryotic flagella, is sensed by the NLRC4 inflammasome [28]. Although *Legionella* reside within vacuoles, it appears that flagellin may still reach the cytosol in a T4SS-dependent manner [29]. NAIP5 binds directly to *Legionella*-derived flagellin by recognizing a C-terminal leucine-rich helical hairpin loop that may be structurally conserved in other PAMPs [30]. Upon binding of flagellin, NAIP5 interacts with NLRC4 to form a functional inflammasome complex and activate caspase-1 [28]. Interestingly, it has been suggested that, upon pathogenic activation of NLRC4, caspase-1-induced pyroptosis and cytokine activation are separable. In response to *Legionella* infection, the canonical NAIP5/NLRC4 inflammasome causes ASC-dependent caspase-1 proteolysis and cleavage of IL-1β, while a distinct NLRC4 inflammasome lacking ASC and containing unprocessed caspase-1 does not cause cytokine activation, but induces pyroptosis [31,32]. In the *Legionella*-permissive A/J mouse line, NAIP5 still interacts with flagellin; however, caspase-1 activation is attenuated by an unknown mechanism [28,30,33]. Caspase-1-dependent restriction of *Legionella* infection still occurs in ASC-deficient macrophages implying that pyroptosis, in addition to cytokine activation, may contribute to limiting *Legionella* infectivity [33,34].

Intracellular pathogens employ various strategies to prevent caspase-1 activation in order to avoid inflammatory cytokine activation, host cell death and neutrophil-mediated clearance. For example, upon phagocytosis, *Salmonella* replace their type III secretion system (T3SS) with an independent T3SS that is no longer recognized by the NLRC4 inflammasome, thus enabling the pathogen to escape immune surveillance [28]. In addition, biofilm-derived *Legionella* demonstrate reduced levels of flagellin expression and, similarly to the genetic deletion of flagellin, this enables them to avoid NLRC4 activation and replicate within otherwise restrictive host cells [29,35]. Flagellated *Legionella* may also downregulate flagellin expression during the intracellular replicative phase, but become flagellated upon amino acid starvation, concomitant with host cell death [36]. However, flagellin-deficient *Legionella* still induce cell death and egress efficiently, suggesting that alternate NLRC4-independent cell death pathways can be activated to promote bacterial release [37].

On the other hand, *Legionella* can also actively suppress the expression of important inflammasome components. For example, in human monocytes and their derived macrophages, which are permissive for *Legionella* replication [38], *Legionella* suppress the protein and mRNA levels of both ASC and NLRC4, but not of caspase-1 and IL-1β [39]. Ectopic expression of ASC in human macrophages allows for caspase-1 activation and reduces *Legionella* growth [39]. NLRC4 may also restrict *Legionella* survival in murine macrophages in a caspase-1-independent manner, particularly in the early stages of infection [40]. The mechanism for this, as well as how *Legionella* specifically downregulate ASC and NLRC4 expression in human, but not murine, macrophages, remains poorly understood. While several murine NAIP paralogs may recognize distinct bacterial proteins (such as PrgJ and flagellin for NAIP2 and NAIP5, respectively) to activate the NLRC4 inflammasome [28,33,41], only one human NAIP ortholog exists [38]. Human NAIP does not appear to activate the NLRC4 inflammasome in response to purified *Legionella* flagellin; however, it can bind to specific subunits of bacterial T3SS, present in *Salmonella,* to activate NLRC4 [28].

• Caspase-11 inflammasome

Caspase-11-deficient mice are defective in caspase-1 activation in response to lipopolysaccharide

(LPS) and are resistant to endotoxic shock [42]. Gram-negative bacteria, such as *Legionella* and *Salmonella*, induce the expression of caspase-11 via a LPS–TLR4–TRIF–Myd88 axis and type I interferon (IFN) production [37,43]. However, LPS activation of caspase-11 occurs in the cytosol and does not require LPS engagement of TLR4 [44,45]. Instead, following its induction, caspase-11 is activated in the host cell cytosol by the penta- and hexa-acyl lipid A moiety of LPS [44,45]. Once activated, caspase-11 induces lytic cell death and IL-1α release, independent of caspase-1, but can also modulate maturation of IL-1β in a process that requires NLRP3 and caspase-1 **(Figure 1)** [46]. Consistent with these findings, caspase-11 is activated upon *Legionella* infection in a NAIP/NLRC4/caspase-1-independent manner to cause pyroptosis, induce IL-1β via NLRP3 and restrict *Legionella* infection *in vivo* [37,47]. How the penta- and hexa-acyl lipid A moieties of LPS are sensed in the host cytosol to cause caspase-11 activation remains unclear, but presumably involves an additional, currently unidentified, receptor [48].

While caspase-11 activation by *Yersinia* or *Legionella* requires functional T3SS and T4SS, respectively [37,47], it is not yet clear whether *Legionella*-derived LPS leaks into the host cytoplasm via the secretion machinery, or if T4SS-competent *Legionella* escape more efficiently owing to membrane rupture. However, it has been observed that other bacterial species, such as *Burkholderia*, which reside primarily within the cytosol, activate caspase-11 following phagolysosomal rupture, as do mutant *Legionella* or *Salmonella* strains that induce vacuole lysis [43].

Caspase-11 may also target bacterial pathogens that reside within specialized compartments by promoting lysosomal fusion [49]. Phagosomes containing virulent *Legionella* rapidly acquire lysosomal markers in wild-type, but not in caspase-11-deficient, macrophages, at the expense of ER-derived vesicles [49]. Notably, however, phagolysosomal maturation is still observed in caspase-11-deficient macrophages harboring avirulent *Legionella* or nonpathogenic *Escherichia coli,* suggesting that the phagolysosomal fusion mediated by caspase-11 is specific to virulent bacteria [49]. There is also some evidence that caspase-11 directly interacts with actin and promotes its polymerization via cofilin [49]. In caspase-11-deficient macrophages, cofilin remains unphosphorylated during infection, in agreement with the lack of actin polymers surrounding *Legionella*-containing phagosomes [49]. Similarly, the human caspase-11 homologs, caspase-4 and caspase-5, promote phosphorylation of cofilin during *Legionella* infection [49]. Whether these processes are directly controlled by caspase-11, or are downstream effects of cell death signaling, awaits further experimental verification. Nevertheless, the above studies indicate that the caspase-11 inflammasome is employed by the host to detect bacteria that reside primarily within the cytosol.

• Caspase-7

In addition to pyroptosis, the NAIP5/NLRC4-mediated activation of caspase-1 promotes the downstream cleavage and activation of caspase-7 [50]. This occurs independently of caspase-3, -8 and -9, and is thus distinct from the caspase-7 activation observed during classical apoptosis. Caspase-7-deficient macrophages and mice also show increased susceptibility to *Legionella* infection [50]. While caspase-3 and-7 fulfill redundant roles during apoptosis, they also show distinct functionality. For instance, in contrast to caspase-7, loss of caspase-3 does not promote intracellular growth of *Legionella* [50]. Also, restrictive macrophages activate caspase-7 upon *Legionella* infection, but do not activate caspase-3, while the opposite is observed in permissive host cells [50]. Caspase-7-deficient macrophages still activate caspase-1 in response to flagellated *Legionella*, ultimately resulting in host cell death [50]. However, there is some evidence that caspase-7 may prevent macrophage death at low levels of infection. Under these conditions, caspase-7 has been shown to trigger phagosome–lysosome fusion leading to reduced bacterial burden [50]. In addition, low numbers of *Legionella* may also induce autophagy via caspase-1, which also results in lysosome-associated bacteria [51]. The molecular mechanism by which caspase-7 is able to modify endocytic trafficking of the LCV remains to be elucidated.

• Autophagy

Autophagy is able to detect and dispose of cytoplasmic content, including proteins, organelles and pathogens, within lysosomes [52]. Intriguingly, flagellated *Legionella* induce higher levels of autophagosomes compared with the flagellin-deficient mutant, suggesting that activating NLRC4 may also trigger autophagy [51]. Conversely, pharmacological inhibition of

autophagy can result in pyroptosis, particularly in macrophages that only harbor a low number of *Legionella.* This suggests that PAMPs may initially trigger autophagy, but, subsequently, induce pyroptosis due to increased/sustained PAMP signaling [51]. Not surprisingly, several *Legionella* effectors target host factors important for autophagy. In particular, RavZ is able to irreversibly deconjugate ATG8, thus effectively blocking the formation of autophagosomes [20].

• Caspase-independent cell death pathways

Several environmental *Legionella* isolates also induce rapid cell death in macrophages [53]. In contrast to the well-studied clinical isolates, these strains fail to replicate in permissive murine, but not human, macrophages, despite the formation of normal LCVs. This is largely because of macrophage cell death that occurs independently of flagellin but requires a functional T4SS [53]. More strikingly, cell death has also been observed in the absence of caspase-1, -11 and -3, as well as in the presence of apoptosis (z-VAD) and necroptosis (necrostatin-1) inhibitors [53]. This suggests the existence of an alternate cell death pathway that is induced in a T4SS-dependent manner by some *Legionella* isolates.

Evasion of inflammatory cell death signals by *Legionella*

• SdhA

The effector protein SdhA is translocated into the host cytosol shortly after *Legionella* uptake and is continually expressed throughout intracellular replication [54]. This 166-kDa protein contains multiple subdomains with largely unknown molecular functions. Besides the secretion signal, the C-terminus contains a canonical GRIP-domain, responsible for protein targeting to the *trans*-Golgi network [55]. The primary function of SdhA is to maintain the integrity of the LCV [56]. Overexpression in HeLa cells results in the formation of novel lipid-bound vacuoles that lack any cellular markers and are similar to those induced by the overexpression of mammalian golgins [55]. Deletion of SdhA alone severely impairs the intracellular growth of *Legionella* and its capacity to cause disease in susceptible mice [57,58]. Macrophages infected with SdhA-deficient *Legionella* show increased nuclear degradation, mitochondrial disruption, membrane permeability and caspase activation [57]. SdhA-deficient *Legionella* also indirectly induce cell death in dendritic cells [59]. In both macrophages and dendritic cells, this phenotype is inhibited neither by overexpression of Bcl-2, nor by the elimination of Bax, Bak or caspase-3, and is consistent with recent findings showing that deletion of SdhA promotes caspase-1- and caspase-11-dependent pyroptosis [43,55,56,59]. This explains the observations:

- That caspase-1 inhibition results in only a partial rescue of bacterial growth in the Δ*sdhA* strain [56], as caspase-1 has been shown to be dispensable for caspase-11-dependent pyroptosis induced by *Legionella* [37];
- That Δ*flaA*/Δ*sdhA Legionella* still induce pyroptosis in the absence of ASC [43].

Phenotypic rescue of the Δ*flaA*/Δ*sdhA* mutant occurs in caspase-11-deficient, but not caspase-1-deficient, macrophages [43] and upon deletion of the *Legionella*-expressed phosphocholine phospholipase, PlaA [56]. In the absence of SdhA, PlaA activity leads to unstable LCV membranes and increased cytosolic localization of *Legionella,* which appears to result in the detection of bacterial DNA and LPS by the AIM2 and caspase-11 inflammasomes to induce pyroptosis [55,56].

The loss of vacuole integrity seen with SdhA-deficient *Legionella* also activates the type I IFN response [60]. Type I IFNs comprise an important class of cytokine that orchestrate diverse innate immune responses to pathogens [61]. The cytosolic presence of bacterial RNA in Δ*sdhALegionella* induces IFN-β expression when compared with wild-type *Legionella*, while removal of PlaA in the Δ*sdhA* strain reduces this effect, although not to wild-type levels. Activation of type I IFNs occurs independently of the flagellin-sensing NAIP5/NLRC4 inflammasome [62], but also appears to contribute to the restriction of bacterial replication within macrophages [63]. While viral suppressors of cytosolic RNA-sensing pathways have previously been recognized, it remains to be determined if *Legionella* effector proteins can directly target host RNA sensing pathways.

Role of apoptotic factors in *Legionella* infection

Macrophages can also respond to pathogenic invasion by initiating the intrinsic mitochondrial apoptotic pathway [64]. While, in general, apoptosis fails to activate inflammatory responses, it can reduce bacterial load, either by blocking the cell cycle of intracellular parasites or, in some cases,

by trapping bacteria within apoptotic remnants [6]. During the early stages of *Legionella* infection, human monocyte-derived macrophages become resistant to apoptosis-inducing agents, such as staurosporine, cycloheximide and TNF-α [65,66]. *Legionella* can also induce expression of several host antiapoptotic genes via activation of the transcription factor NFκB, while the pharmacological inhibition of NFκB induces apoptosis and impairs *Legionella* growth [65,67]. It is only at the late stages of infection that macrophages show increasingly apoptotic phenotypes, such as nuclear condensation and phosphatidylserine exposure [65,68]. While little is known about how *Legionella* may induce apoptosis during this period, it appears to include signals that operate independently of the essential mitochondrial apoptosis components Bax and Bak [59].

Legionella infection in other immune cells

While macrophages represent the principal mammalian host cell for intracellular growth of *Legionella*, these bacteria also interact with other immune cells, including dendritic cells. Similarly to macrophages, dendritic cells (including plasmocytoid dendritic cells) are recruited in the early stages of *Legionella* infection in mice [9]. Although able to phagocytose *Legionella*, *in vivo* infections in mice have demonstrated that dendritic cells contain only a few bacteria at a time. For the most part, murine dendritic cells make poor hosts for *Legionella* replication owing to the rapid onset of cell death that follows infection [59]. *Legionella*-induced dendritic cell death is completely abrogated in the absence of caspase-3 or Bax/Bak, and is also reduced upon expression of the antiapoptotic protein Bcl-2. Furthermore, caspase-3 and, particularly, Bax/Bak-deficient dendritic cells are able to sustain *Legionella* growth over several days, demonstrating that mitochondrial apoptosis is the primary mechanism of dendritic cell death caused by *Legionella* infection [59]. This suggests that, unlike *Legionella*-infected permissive macrophages, murine dendritic cells fail to upregulate antiapoptotic factors [65]. Surprisingly, apoptosis of *Legionella*-infected dendritic cells is also dependent upon HABP1, p32 and gC1qR [69]. It is known that the mitochondrial-localized p32 subunit is able to recruit other factors that sensitize host cells to apoptosis [70]. Interestingly, the closely related pathogen *Coxiella burnetii* expresses an ankyrin repeat effector, AnkG, which specifically binds p32 to block apoptosis in dendritic cells [69]. There is only limited evidence demonstrating that human primary dendritic cells also undergo cell death [71]. In this case, cell death is sufficiently delayed to allow for some replication of *Legionella* [71]. However, little is known about whether human dendritic cells also induce canonical apoptosis, as seen in murine cells. Furthermore, it will be important to test whether cell death in *Legionella*-infected dendritic cells prevents the spread of *Legionella* bacteria to other organs.

Legionella effectors that modulate apoptotic responses

• VipD (LegS2, Lem12, Ceg18 & Lpg0716)

It is possible that *Legionella* actively induce apoptosis in dendritic cells to prevent maturation and, hence, activation of adaptive immune responses in the lymphatic system. Given that *Legionella*-infected dendritic cells activate caspase-3 to induce cell death, Zhu *et al.* recently screened 275 effectors for caspase-3 activation in 293T cells [72]. This screen identified five candidates (VipD, LegS2, Lem12, Ceg18 and Lpg0716) that share little sequence similarity. At least three of these (LegS2, Lem12 and Ceg18) are targeted directly to mitochondria and may promote caspase-3 activity, mitochondrial damage, cytochrome c release and apoptosome formation **(Figure 1)**. VipD is a putative phospholipase A2 and localizes to the plasma membrane, cytoplasm, and nucleus when expressed as a GFP-chimera in HeLa cells [72]. There is some evidence that VipD targets at least two major phospholipids (phosphatidylcholine and phosphatidylethanolamine) and that the lysoproducts and/or free fatty acids generated by VipD activity can cause mitochondrial membrane damage [72]. While phospholipases can have broad specificity, VipD only affects the integrity of mitochondria and not the plasma membrane. This could be partially explained by the fact that VipD only exerts its lipase activity in the presence of a mitochondrial proteinaceous factor. *Legionella* also targets other lipid classes, as the effector LegS2 is a sphingosine-1-phosphate lyase [72,73]. While sphingolipids mediate apoptosis and LegS2 expression leads to caspase-3 activity, the role of LegS2 during infection remains uncharacterized.

• SidF

Thus far, SidF is the only *Legionella* effector known to target and inhibit host proteins

implicated in cell death. *Legionella* lacking SidF show reduced growth rates in macrophages, resulting in a lower number of vacuoles containing more than ten bacteria [74]. Initially, SidF was found to bind and inhibit two mammalian host cell factors, termed BNIP3 and Bcl-rambo [74]. BNIP3 is a putative proapoptotic BH3-only protein that has been implicated in the modulation of apoptosis, autophagy and necrosis [75]. Bcl-rambo can induce cell death when overexpressed, and has recently been proposed to interact with ANT, the ADP and ATP transporter of mitochondrial membranes, to induce loss of mitochondrial membrane potential and caspase activation [76]. Unlike other prodeath factors, BNIP3 and Bcl-rambo kill cells via a C-terminal transmembrane domain [75,76]. Interestingly, SidF inhibition of BNIP3 and Bcl-rambo depends upon two hydrophobic domains that may constitute transmembrane regions important for ER/LCV targeting [77,78]. Whether these regions directly block the C-terminal domains of BNIP3 and Bcl-rambo, required for their targeting to mitochondria, awaits further proof. Notably, a recent study has identified a phosphatidylinositol polyphosphate 3-phosphatase domain in SidF that specifically hydrolyzes $PI(3,4)P_2$ and $PI(3,4,5)P_3$ [77]. The latter is enriched in LCV membranes and is an important anchoring signal for other effectors. Given that *Legionella* actively targets host cell lipids [73], it remains possible that this strategy also prevents activation of cell death and inflammatory signaling pathways [23].

- **Ncp**

We have recently demonstrated that *Legionella* export an ATP-specific Ncp via the T4SS, which is targeted to the inner mitochondrial membrane in macrophages [78]. Host ATP/ADP carriers are thought to play a critical role in apoptosis by compromising the bioenergetics of mitochondria, resulting in cytochrome c release and apoptosome formation [79]. Surprisingly, while *Legionella* Ncp behaves biochemically as an ATP transporter, it fails to compensate yeast mutants lacking endogenous carrier proteins, suggesting unique properties [78]. Indeed, unlike the canonical ATP transporter, Ncp behaves predominantly like an exporter of ATP and thus may regulate mitochondrial bioenergetics under certain conditions. For example, overexpression of Ncp is toxic in yeast grown in nutrient-limiting media [78]. This suggests that Ncp is able induce cell death on its own under certain conditions, particularly when mitochondrial ATP import is essential. Similar to other effectors, loss of Ncp does not abrogate intracellular *Legionella* growth, suggesting other effectors may fulfill a similar role.

Conclusion

Macrophages employ several innate immune responses to combat invading pathogens, including the induction of cell suicide programs. Despite this, *Legionella* are able to survive within permissive macrophages for extended periods, with bacterial replication often occurring between 5 and 18 h after invasion and with little host cell death occurring during this time [80]. Unlike other intracellular pathogens that often show reduced genome sizes, *Legionella* has opted to increase its effector repertoire, likely to ensure survival within a broad range of host cell types (including protozoan *Amoebae* and mammalian immune cells). Furthermore, several *Legionella* effectors have been retained that fulfill functionally redundant roles, although they utilize different molecular mechanisms. By unraveling the function of these effectors, novel macrophage innate immune responses have been and undoubtedly will be uncovered.

Future perspective

The vast majority of *Legionella* effectors remain uncharacterized, hampered by their low protein homology to known sequences and, at best, mild phenotypes upon genetic deletion. Novel screening methods are needed to test their roles during the infection of diverse host organisms (successfully employed in [81]). In particular, it is now clear that *Legionella* can activate multiple cell death pathways, each of which may promote bacterial clearance. As with other signaling pathways, there is considerable cross-talk between different cell death factors, often to ensure adequate responses [1]. Thus, it is likely that *Legionella* have evolved multiple mechanisms to avoid or modulate macrophage death during intracellular replication. In addition, data are now emerging demonstrating the ability of pathogens to activate cell death factors to their own advantage, in particular during escape from macrophages [82]. Notably, bacteria must ensure the specificity of the cell death signal, as apoptosis of macrophages prevents reinfection by *M. tuberculosis,* whereas necrosis

promotes it [6]. While permissive macrophages show hallmarks of apoptosis during egress of *Legionella*, there is a lack of genetic evidence that canonical apoptotic factors play a major role during infection. By contrast, dendritic cells clearly utilize the apoptotic machinery to induce rapid cell death in response to *Legionella* [59]. Whether this is true for other immune cells, such as neutrophils, remains to be seen. In addition, we know little regarding the role of cell death factors during *Legionella* infection in animal models. In particular, novel imaging methods are needed to visualize the cellular bacteria–host interactions in more detail. However it is clear that pharmacological manipulation of host cell death signaling during bacterial infections may represent an important strategy for the development of new antibacterial drugs.

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EXECUTIVE SUMMARY

Legionella

- *Legionella* are Gram-negative bacteria that survive in aquatic environments resisting digestion by *Amoebae*.
- *Legionella* are transmitted to humans via contaminated aerosols generated by cooling towers, water fountains and water heaters.
- *Legionella* infections may result in severe, inflammatory pneumonia, which primarily affects elderly and immunocompromised individuals (Legionnaires' disease), or in a milder respiratory illness (Pontiac fever).
- In the lungs, *Legionella* survive within alveolar macrophages in the long term by suppressing host innate immune responses.

Legionella effectors

- *Legionella* contain a type IV secretion system, essential for survival in *Amoebae*, macrophages and susceptible mice strains.
- The type IV secretion system exports 295 effectors to the host cell that target diverse factors and organelles.
- Effectors show little sequence homology to known proteins and contain considerable functional redundancy.
- Effectors may target several host factors within a common or redundant pathway, including innate immune responses.

Host cell death responses

- Macrophages recognize, engulf and digest microbes, and regulate adaptive immunity in order to combat infectious agents.
- Invading pathogens are sensed via pathogen-associated recognition patterns (PAMPs).
- PAMPs trigger the release of proinflammatory signals via large cytosolic complexes (termed inflammasomes) and induce cell death responses.

Macrophage–*Legionella* interaction

- *Legionella* have evolved several mechanisms to avoid innate immune recognition and activation of cell death pathways.
- Several effectors regulate phagosome maturation, resulting in a unique vacuole that protects from cytosolic pathogen sensors.
- Vacuolar-released PAMPs and cytosolic *Legionella* activate several inflammasomes.
- *Legionella* suppress canonical apoptotic signals in macrophages, but not dendritic cells.

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CHAPTER 2
MATERIALS AND METHODS

2.1 Bacterial strains

Legionella pneumophila 130b serogroup 1 (ATCC BAA-74) is a spectinomycin-resistant clinical isolate from the Wadsworth Veterans Administration Hospital, Los Angeles, CA (Edelstein, 1986). The avirulent Δ*dotA* strain, the Δ*icmS* strain, and the flagellin-deficient Δ*flaA* and Δ*flaA*/Δ*sidF* strains are all deletion mutants of *L. pneumophila* 130b (Harding *et al.*, 2013). The Δ*flaA*/Δ*5* and Δ*flaA*/Δ*7* strains were generated on the *L. pneumophila* LP02 background, a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The *L. longbeachae* NSW-150 strain is a serogroup 1 clinical isolate from Australia (Cazalet *et al.*, 2010). In the absence of an avirulent *Longbeachae* control strain, WT *L. longbeachae* were heat-killed at 85 °C for 10 min. In some strains, GFP was expressed constitutively using the plasmid pMIP:GFP (Riedmaier *et al.*, 2014).

2.1.1 Generation of Legionella mutants and their complemented strains

The Δ*dotA* deletion strain was generated as outlined in (Berger *et al.*, 1994). The Δ*flaA* deletion strain was generated as outlined in (Merriam *et al.*, 1997; Harding *et al.*, 2013). The Δ*flaA*/Δ*5* and Δ*flaA*/Δ*7* mutants were generated from LP02 by sequential in-frame deletion using the suicide plasmid pSR47S as described in (Shen *et al.*, 2009). The Δ*icmS* strain is a markerless deletion mutant also generated using pSR47s. To generate the Δ*flaA*/Δ*sidF* deletion strain, ~ 500 bp fragments from upstream and downstream of sidF (LPW_28321) were amplified with the primers 2842/2843 and 2844/2845 (Table 2.1) and fused together using overlap extension (OE) PCR. The

construct was cloned into the Sal1 site of the plasmid pSR47S, and Δ*flaA L. pneumophila* were transformed to select for kanamycin resistant clones. The second integration to delete the entire *sidF* coding region was selected for on 10 % sucrose plates. Individual colonies were verified for loss of *sidF* using the primers 2841 and 2846 to amplify the *sidF* genomic region.

Table 2.1 Primers used in this thesis

Constructing the Δ*flaA*/Δ*sidF* deletion strain

Primer name[1]	**Gene/ORF**	**Sequence 5' – 3'**
2841	SidF	GCTCAGCAAATTATCAATACAGC
2842	SidF	AGCTAGGTCGACACAGCGCTCTTATCTCAAGTTC
2843	SidF	CTATATTTTCAGTGATTCGTGGCATGG
2844	SidF	CCATGCCACGAATCACTGAAAATATAGCAGTAAACT TCTAAGGGTTATGAG
2845	SidF	AGCTAGGTCGACGTTCCTGTATCGATCGAATCGTC
2846	SidF	GTGTTTATTCAGCCTGGAATAATC

Constructing the complemented Δ*flaA*/Δ*sidF strains*

Primer name	**Gene/ORF**	**Sequence 5' – 3'**
100	SidF	TCCCCCGGGGAATGCCACGAATCACTGAAAATATG
101	SidF	CGGGATCCTTAGAAGTTTACTGGCGTGGGGGTG
102	SidF	TCCCCCGGGCAGGGCCTGAAAACGCTTTTA
104	Sequencing	CACACAGGAAACAGAATTCAG
105	Sequencing	CTGTATCAGGCTGAAAATCTT
106	Sequencing	ATCCAAACCCTGGCACAG
107	Sequencing	GAGCTCTTGAGCTTGCATGA

[1] Numbers (#) refer to the Naderer Lab primer database reference numbers

The complemented Δ*flaA*/Δ*sidF* strains were made by cloning the full-length sidF gene (FL-SidF) or the C-terminal region (CT-SidF), comprising base pairs 2232 to 2379 (amino acids 744 to 912), into the plasmid pMMB207C. The primers 104, 105, 106, and 107 were used to confirm insertion of the correct sequence. A control strain containing only the empty pMMB207C vector was also constructed. The primers used to amplify the full-length SidF construct were 100 and 101. The primers used to amplify the C-terminus of SidF were 101 and 102. The PCR programme used is outlined in Table 2.2.

Table 2.2 PCR programme used to amplify SidF constructs

Step	Temperature (°C)	Time (min)	Repeat
1	94	2	1
2	94	0.5	26
3	55	0.5	
4	72	3	1
5	72	5	1
6	4	∞	1

2.1.2 Growth of Legionella strains

Legionella strains were grown at 37 °C from –80 °C frozen glycerol (60 %) stocks on buffered charcoal-yeast extract (BCYE) agar for 48 h before each infection. To make BCYE agar, 12.5 g of *Legionella* CYE agar base (Oxoid) was added to 450 mL distilled H_2O and 50 mL 10 % N-(2-Acetamido)-2-aminoethane sulfonic acid (ACES) (pH adjusted to 6.9 with KOH). This solution was sterilised by autoclaving and allowed to cool to approximately 50 °C before addition of 3.3 mM *L*-cysteine and 0.33

mM $Fe(NO_3)_3$. Chloramphenicol was included at 6 μg.mL^{-1} to select for strains expressing GFP, or complemented strains. Thymidine was added at 100 μg.mL^{-1} for the thymidine auxotroph strains. To determine bacterial numbers before infection, an inoculation loop full of *Legionella* was scraped from the plate, re-suspended in 1 mL phosphate-buffered saline (PBS), and the optical density at 600 nm (OD_{600}) determined. An OD_{600} of 1 was taken to be equal to 10^9 bacteria/mL. Further dilutions were then made up in PBS to reach an appropriate multiplicity of infection (MOI) (Table 2.3).

Table 2.3 MOIs used in different experiments

Experiment	Cell type	MOI
Live-cell imaging	BMDMs	5, 10, 20
	THP-1-derived macrophages	5
FACS	C57BL/6 immortalised macrophages	10*, 50*
CFUs	BMDMs	10, 50*
	THP-1- derived macrophages	5*
Westerns	BMDMs	10, 25‡, 50‡
Immunofluorescence	BMDMs	10
Radio-labelling	BMDMs	25‡, 50‡

* Medium was replaced after 2-4 h.

‡ Plates were spun at 250 ×*g* for 5 min at 4 °C, and the medium replaced after 2 h.

2.2 Culture of macrophages

2.2.1 Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were obtained from femora and tibiae of female 6-8 week-old C57BL/6 mice, or from mice of the indicated genotypes. Bone

marrow was flushed from the bones using a 26G × ½ in. needle and 9 mL of BMDM culture medium, comprising RPMI 1640 medium (Gibco) supplemented with 15 % foetal bovine serum (Serana), 20 % L-cell-conditioned medium (containing macrophage colony-stimulating factor), and 100 U/mL of penicillin-streptomycin (Sigma). Cells were then placed in a T25 flask overnight at 37 °C + 5 % CO_2, allowing the adherent cells to be separated from the non-adherent monocytes. Monocytes were then transferred to bacteriological dishes (8 per mouse) and differentiated in BMDM culture medium for 7-12 days, at 37 °C + 5 % CO_2. For culture periods longer than 7 days, the medium was replaced after one week. To seed BMDMs into multi-well plates, 24 h before infection cells were gently scraped from petri dishes using a cell scraper (BD Falcon) and washed 3 times in PBS to remove the penicillin-streptomycin. Cells were seeded into tissue culture-treated plates (Corning or BD Falcon) at a density of 4.5×10^5 cells/mL in order to create an ~90 % confluent monolayer.

2.2.2 Immortalised C57BL/6 mouse-derived macrophages

Immortalised C57BL/6 mouse-derived macrophages (Bauernfeind *et al.*, 2009) were cultured in RPMI 1640 supplemented with 10 % foetal bovine serum, in bacteriological dishes, at 37 °C + 5 % CO_2. To seed plates, 24 h before infection BL/6 cells were removed from petri dishes using a 10 mL pipette and seeded into tissue culture-treated plates (Corning or BD Falcon) at a density of 2×10^5 cells/mL. At this density, cells reached 100 % confluency within 24 – 36 h and were still impermeable to propidium iodide (PI), *i.e.*, healthy, after 48 h.

2.2.3 Human THP-1-derived macrophages

Human THP-1 monocytes were cultured in RPMI 1640 supplemented with 10 % foetal bovine serum, in T75 flasks, at 37 °C + 5 % CO_2. To seed plates, 48 h before infection

monocytes were seeded into tissue culture-treated plates (Corning or BD Falcon) at a density of 1×10^6 cells/mL, and differentiated into macrophages using 160 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma) over 24 h. The medium was then replaced and macrophages were left to recover for an additional 24 h before infection.

2.3 Quantification of *Legionella* infection *in vitro*

2.3.1 Live-cell imaging

To follow *Legionella* infection in real-time using live-cell imaging, macrophages were seeded into 96-well tissue culture-treated plates. Before infection, BMDMs were stained with 1 μM Cell Tracker Green (CTG) (Invitrogen) for 20 min in serum-free RPMI 1640, and THP-1-derived macrophages were stained with 1 μM CTG for 10 min in serum-free RPMI 1640. Medium was then replaced with RPMI 1640 supplemented with 15 % FBS and 10 % L-cell-conditioned medium containing 50 nM tetramethylrhodamine (TMRM) and 600 nM Draq7 (Abcam). Cells were then infected with *Legionella* strains at a range of MOIs: 5, 10, or 20 for BMDMs, and 5 for THP-1 cells (Table 2.3). Before imaging, 50 μL of mineral oil (Sigma) was added to each well to prevent evaporation.

Experiments were performed on a Leica AF6000 LX epi-fluorescence microscope equipped with an incubator chamber set at 37 °C + 5 % CO_2 and an inverted, fully-motorised stage driven by Leica Advanced Suite Application software. Time-lapse images were acquired with bright-field, GFP, TxRed, and Y5 filters every hour for up to 72 h using a 10×/0.8-A objective. To determine the percentage of dead cells, images were analysed in ImageJ and in MetaMorph (Molecular Devices) using a custom-made journal suite incorporating the count nuclei function to segment and count the number

of CTG and Draq7-positive cells (adapted from Croker et al. (Croker *et al.*, 2011). Due to bleaching of the CTG in experiments running longer than 24 h, only the first frame of the GFP channel was used to calculate total cell number. The data of percentage Draq7-positive cells were analysed in Excel and GraphPad Prism.

2.3.2 Flow cytometry analysis

To determine bacterial burdens using flow cytometry, immortalised C57BL/6 mouse-derived macrophages were seeded into 12-well tissue culture-treated plates at a density of 2×10^5 cells/mL, and infected with *Legionella* strains expressing GFP at a MOI of 10 or 50 (Table 2.3). After 2 h, cells were washed 3× in PBS and the medium replaced. If required, at this point cells were treated with 500 nM or 1 μM of BH3 mimetics and/or 20 μM of the pan-caspase inhibitor (3S)-5-(2,6-Difluorophenoxy)-3-[[(2S)-3-methyl-1-oxo-2-[(2quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate (Q-VD-OPh) (R&D Systems), and/or 500 $ng.mL^{-1}$ of *P. aeruginosa* Exotoxin A (Sigma).

To harvest cells for analysis, the medium was replaced with 0.5 mL of 50 mM ethylenediamine tetra-acetic acid (EDTA) made up in PBS, for 10 min, at room temperature. At later time-points (> 24 h), the supernatant was also collected and centrifuged at 211 × *g* to collect any cells that had disassociated from the plates. Following treatment with EDTA, a P1000 pipette was used to gently remove the cells to microcentrifuge tubes, which were pelleted at 211 × *g* for 5 min. The supernatant was removed and discarded, and the cell pellets re-suspended in 300 μL of PBS containing 5 mM EDTA to prevent cells from clumping, and 1 $\mu g.mL^{-1}$ propidium iodide (PI) (Invitrogen). A PI-negative sample was also prepared as a control. GFP and PI fluorescence were then determined by flow cytometric analysis using a BD

FACSCalibur. Cells were gated by forward and side scatter, and channels F1 and F3 were used to detect GFP and PI fluorescence, respectively. 10,000 gated events per sample were counted. WEASEL software (WEHI) was used for the analysis.

2.3.3 Measurement of colony-forming units (CFUs)

To determine bacterial burdens by counting CFUs, BMDMs were seeded in triplicate into 24-well tissue culture-treated plates at a density of 4.5×10^5 cells/mL, and infected with *Legionella* strains at a MOI of 10 (medium left on) or 50 (medium replaced after 4 h without washes) (Table 2.3). If required, at this point cells were treated with BH3 mimetics [500 nM] and/or Q-VD [20 μM].

To harvest cells, macrophage medium was collected in microcentrifuge tubes and the remaining cells were lysed in 200 μL of 0.05 % digitonin in PBS for 5 min at room temperature. Cell lysates were then pooled with, or analysed separately from, the culture media to obtain the total number of CFU, or only the macrophage-associated CFU, respectively. Serial dilutions of the cell lysates from 10^{-1} to 10^{-5} were generated in microcentrifuge tubes by removing 20 μL from each previous tube and placing it in 180 μL of PBS. 20 μL of each dilution was then plated drop-wise onto BCYE agar plates and allowed to run down the plate, forming a column (4× 20 μL drops per plate). Bacterial colonies were counted after 72 h at 37 °C.

2.4 Mice infections

6-8 week-old male or female C57BL/6 and A/J mice (or mice of the indicated genotypes), in groups of five or more, were anesthetised by 5 % isofluorane inhalation for 3-5 min to induce a moderate state of unconsciousness. Mice were then infected intra-nasally using a P200 pipette to drip 50 μL of sterile PBS containing either 2.5 ×

10^6 *L. pneumophila*, or 1×10^5 *L. longbeachae*, onto the nose whilst holding the mouse loosely by the scruff of the neck in a vertical position. Mice were monitored until fully recovered.

2.5 Intra-peritoneal injection

In some instances, after intra-nasal infection, mice were injected intra-peritoneally (i.p.) with 50 $mg.kg^{-1}$ body weight of ABT-263 (Selleckchem), ABT-199 (WEHI), or a BCL-XL-specific inhibitor (WEHI), made up in 50 μL of DMSO, or with DMSO (as a vehicle control) only. For the ABT-263 studies, the analysing investigator was blinded towards which group received the BH3 mimetic (no other studies were blinded).

2.6 Quantification of *Legionella* infection *in vivo*

2.6.1 Measurement of colony-forming units (CFUs)

To determine bacterial lung loads, mice were euthanised via inhalation of CO_2 at 72 h post-infection. Both lung lobes were removed and homogenised for 20-30 s in 2 mL of PBS at 30 000 rpm, using the Omni Tissue Master homogeniser. Serial dilutions of the lung homogenates were made up in microcentrifuge tubes by removing 20 μL from each previous tube and placing it in 180 μL of PBS. 20 μL of each dilution was then plated drop-wise onto BCYE agar plates and allowed to run down the plate, forming a column (4× 20 μL drops per plate). Bacterial colonies were counted after 72 h at 37 °C.

2.6.2 Survival

Mice used for survival experiments were given a minimum of 3 days to recover from transport in order to ensure weight stabilisation. Following infection, mice were

weighed daily at the same time and euthanised after > 15 % weight loss was observed, according to Monash animal ethics guidelines.

2.6.3 Enzyme-linked immunosorbent assay (ELISA)

Mice were euthanised via inhalation of CO_2 at 72 post-infection. Both lung lobes were removed and homogenised for 20-30 s in 2 mL of PBS at 30 000 rpm, using the Omni Tissue Master homogeniser. Lung lysates were kept at – 80 °C until use. For TNF-α, the Mouse TNF-α ELISA Ready-SET-Go® kit by Affymetrix eBioscience (CAT# 88-7324) was used according to the manufacturer's instructions. For IL-1*β*, the Mouse IL-1 beta/IL-1F2 DuoSet by R&D Systems (CAT# DY401) was used according to the manufacturer's instructions. For both kits, volumes of reagents used were halved.

2.7 *Bcl-x*-deficient mice strains

2.7.1 Cre-mediated Bcl-x deletion

Constitutive loss of BCL-XL results in embryonic lethality around E14 due to excessive apoptosis of erythroid and neuronal progenitors (Motoyama *et al.*, 1995). Therefore, to generate *Bcl-x*-deficient mice we used Bcl-$x^{flox/flox:}$;ER-Cre mice, which contain two floxed *Bcl-x* alleles (Wagner *et al.*, 2000) and express the Cre-recombinase oestrogen receptor fusion protein (Rosa26-Cre-ERT2) upon treatment with tamoxifen.

To induce Cre-mediated *Bcl-x* deletion in mice, 3 doses of 200 $mg.kg^{-1}$ tamoxifen (Sigma, T5648), dissolved in peanut oil and 10 % ethanol (at 80 $mg.mL^{-1}$), were administered over one week on three separate days. On each occasion, mice were scruffed and held vertically, whilst fully conscious, before 50 μL of tamoxifen was delivered via oral gavage using bulb-tipped feeding needles. Mice were then left for 6-

8 weeks before infection with *Legionella* in order to allow time for the gene knock-out to take effect, as well as for the bone marrow to fully recover from the tamoxifen treatment.

To induce Cre-mediated *Bcl-x* deletion in macrophages, bone marrow progenitor cells from Bcl-$x^{flox/flox:}$;ER-Cre mice were extracted as outlined in *2.2.1*. Cells were plated into bacteriological dishes and treated with 50 nM 4-hydroxy tamoxifen (4-HT) dissolved in ethanol after 2 days. *In vitr*o and *in vivo* deletion of *Bcl-x* was confirmed by Western blotting.

2.7.2 LysM cre Bcl-x deletion

In order to generate a meyloid-specific *Bcl-x* knock-out mouse, *Bcl-*$x^{flox/flox}$ mice (Wagner *et al.*, 2000) were crossed with *LysMcre* mice (Clausen *et al.*, 1999). Littermate *Bcl-*$x^{flox/flox}$*; LysM*$^{+/+}$ mice were used as internal controls in all experiments. To confirm that deletion of BCL-XL in myeloid cells had no overt phenotype, leukocyte counts and peripheral blood cells from cardiac bleeds were counted on an Advia 120 cell analyser (Bayer Diagnostics).

2.8 Immuno-blot analysis

Macrophages were seeded into 24 well tissue culture-treated plates at a density of 4.5 × 10^5 cells/mL and infected with *Legionella* strains at a MOI of 10. To harvest the cells, medium was removed from wells and 120 μL of 1× SDS-loading dye (40 % glycerol, 8 % SDS, 200 mM DTT, 40 mM EDTA, 0.0 5% bromophenol blue, and 250 mM Tris-HCl [pH 6.8]) added. Cell lysates were then kept in microcentrifuge tubes at –80 °C until required.

Before analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were boiled for 5 min. Depending upon the size of the proteins to be analysed, 10-15 % bis-acrylamide (Bio-Rad) separating gels were used (made in-house) (Table 2.4). Each gel had a stacking layer (Table 2.5). Gel electrophoresis was performed at 200 V for ~ 45 min, or until the dye front had run off the gel. The SDS-PAGE running buffer contained 25 mM Tris, 192 mM glycine and 1 % SDS. Proteins were then wet-transferred at 4 °C to nitrocellulose membranes (Millipore) at 100 V, for 60 min, using a buffer containing 380 mM glycine, 202 mM Tris, and 10 % methanol. Ponceau staining was used to confirm the transfer and to check protein levels in each lane.

Membranes were blocked with 5 % skim milk in T-BST (0.2 % Tween-20, 137 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 7.4) for 1 h at room temperature. Membranes were then probed with anti-cleaved caspase-3 antibody (CST #9964), anti-BCL-XL antibody (WEHI and CST #2764), anti-BCL-2 antibody (CST #2875), anti-MCL-1 antibody (WEHI and CST #5453), anti-β-actin antibody (Millipore #04-1116), or anti-F1-β antibody (Monash Antibody Facility) and re-suspended in T-BST and 5 % skim milk overnight, at 4 °C. β-actin and F1-β were used as loading controls. After washing, membranes were probed with secondary goat anti-rabbit IgG (Life Technologies), goat anti-rat IgG (Life Technologies), or goat anti-mouse IgG (Life Technologies) antibodies conjugated to horse radish peroxidase (HRP) (1:20 000 dilution in T-BST + 5 % skim milk). Membranes were developed with the luminol-based enhanced chemiluminescence (ECL) reagent (made in-house), before exposure to film (Kodak). Scanned images were then processed using Adobe Photoshop and Adobe Illustrator.

Table 2.4 SDS-PAGE stacking gels

Separating Gel	**2 × 15 %**	**2 × 12 %**	**2 × 10 %**
H_2O	3.73 mL	4.48 mL	4.98 mL
40 % bis-acrylamide	3.75 mL	3.0 mL	2.5 mL
Separating buffer*	2.52 mL	2.52 mL	2.52 mL
20 % ammonium persulfate (APS)	50 μL	50 μL	50 μL
TEMED	6.0 μL	6.0 μL	6.0 μL
TOTAL	**~10 mL**	**~10 mL**	**~10 mL**

*Separating Buffer: 1.5 M Tris, 0.4 % SDS, 2mM EDTA, pH 8.8

Table 2.5 SDS-PAGE separating gels

Stacking Gel	**Volume**
H_2O	2.94 mL
40 % bis-acrylamide	0.525 mL
Stacking buffer*	0.525 mL
20 % APS	42 μL
TEMED	4.2 μL

*Stacking Buffer: 1 M Tris, 0.08 % SDS, 2 mM EDTA, pH 6.8

2.9 Immunofluorescence staining

Macrophages were seeded onto glass coverslips in 24-well plates at a density of 2.25 × 10^5 cells/mL and infected with *Legionella* strains expressing GFP at a MOI of 10. If required, BH3-mimetics [500 nM] were added to appropriate wells 2 h after infection. At specific time-points, cells were fixed with 300 µL of 4 % paraformaldehyde (PFA) for 15 min at 37 °C + 5 % CO_2, washed three times with PBS, and treated with 300 µL of 50 mM NH_4Cl for 10 min. Cells were then permeabilised in 0.1 % Triton-X 100 in PBS for 5 min on ice, and blocked in PBS + 1 % bovine serum albumin (BSA) overnight, at 4 °C. Coverslips were incubated with anti-cleaved caspase-3 antibody (CST #9964) [1:400], for 30 min, at room temperature. After three washes in PBS, cells were incubated with goat anti-rabbit IgG antibodies coupled to Alexa Fluor 568 (Life Technologies) [1:1000] and/or goat anti-mouse IgG antibodies coupled to AlexaFluor 647 (Life Technologies) [1:1000] for 30 min. Cells were mounted in oil (Dako) containing 10 µg.mL^{-1} Hoechst 33342 (Life Technologies) and imaged on an Olympus epi-fluorescence microscope using a 60× oil objective (0.8A). Images were analysed in ImageJ.

2.10 [^{35}S]methionine-labelling of proteins

Macrophages were seeded into 24-well tissue culture-treated plates at a density of 4.5 × 10^5 cells/mL and infected with *Legionella* strains at different MOIs (Table 2.3). Twenty-five minutes prior to labelling, cells were treated with 25 µg.mL^{-1} chloramphenicol to inhibit bacterial protein translation. At 6 and 24 h post-infection, medium was removed and cells were incubated with 25 µCi/mL [^{35}S]methionine (Perkin Elmer) in RPMI 1640 medium without methionine and supplemented with 10

% FBS, 2 mM l-glutamine, 25 μg/ml chloramphenicol, and 10 % L929 cell supernatant. Cells were labelled for 1 h, washed three times with cold PBS, and then lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 1× Roche protease inhibitor mixture (no EDTA) (pH 8.0) for 20 min at 4 °C. Total protein levels were measured by bicinchoninic acid (BCA) assay, and equal amounts of protein were mixed with 1× SDS loading dye, boiled for 5 min, and then separated by SDS-PAGE. Finally, the gels were stained with Coomassie blue to show equal protein loading, dried, and then exposed to a phosphor screen and visualised using a Typhoon Trio imager (GE Healthcare).

2.11 Measurement of cell death following translation inhibition

Seven day-old WT, MCL-1-fN, or *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs were treated with 20 μg.mL^{-1} of cycloheximide (Sigma), 500 nM episilvestrol (WEHI), or 500 ng.mL^{-1} of Exotoxin A (Sigma) alone, or in combination with 20 ng.mL^{-1} ultrapure lipopolysaccharide (LPS) (Invivogen), and 1 μM etoposide (VP16) (Clifford Hallam) for 5 or 18 h. Death was measured by propidium iodide (PI) staining and flow cytometric analysis (see *2.3.2*), before quantification using WEASEL software (WEHI).

2.12 Statistical analyses

For all *in vitro* data, 2-way ANOVA was performed before using Tukey's post-hoc test for pair-wise comparisons. In all experiments, p values ≤ 0.05 were taken to be significant. In Chapter 4, for figure 4b the Mann-Whitney U test was used. For the analysis in Chapter 4, figures 4c and d, the independent samples were bootstrapped to test for the difference between CFUs in treated and untreated mice. This confirmed

that CFUs were significantly greater in the non-treatment condition (99 % CI for difference between the two sample groups). In all cases, the lower bound of the confidence interval for the difference between conditions was substantially different to zero (in the expected direction), confirming that the conditions were significantly different, $p < 0.01$.

CHAPTER 3

THE ROLE OF CELL DEATH PATHWAYS IN *LEGIONELLA* INFECTION

3.1 Introduction

As outlined in Chapter One, the *Legionella*-macrophage interaction involves the activation and/or repression of a number of host cell death pathways. To date, many of the biochemical processes that lead to the establishment of the *Legionella*-containing vacuole (LCV) have been elucidated [reviewed in (Isberg *et al.*, 2009; Xu and Luo, 2013)], however, little is still known about how host cell factors contribute to the integrity of the mature LCV during the replicative and egress phases of infection. Once resources are depleted, *Legionella* must initiate escape from the LCV and its host cell in order to perpetuate infection. However, the mechanism by which *Legionella* induce this egress is poorly characterised.

Molmeret et al. have reported that *Legionella* rupture the LCV membrane and escape to the cytosol, where they complete their terminal rounds of proliferation (Molmeret *et al.*, 2004a; Molmeret *et al.*, 2004b). In this cytosolic, terminal phase of replication, *Legionella* up-regulate several genes associated with pore-forming toxins and motility, including flagellin (Molmeret *et al.*, 2010). Although some studies have suggested that *Legionella* might use pore-forming toxins to egress from cells (Terry Alli *et al.*, 2000; Molmeret and Abu Kwaik, 2002; Molmeret *et al.*, 2002), there is little biochemical evidence for this. A 32 amino acid truncation at the C-terminus of the type-4 secretion system (T4SS) effector protein, icmT, results in *Legionella* that are defective in pore

formation-mediated cytolysis and egress from both protozoan and mammalian cells (Molmeret *et al.*, 2002; Molmeret *et al.*, 2002). However, no biochemical or molecular mechanism for icmT function has ever been elucidated (Bitar *et al.*, 2005). An alternative possibility that remains relatively unexplored is that intracellular *Legionella* induce host macrophage cell death pathways, *e.g.*, apoptosis or pyroptosis, in order to aid in their egress.

Throughout the initial and replicative phases of infection, *Legionella*-infected macrophages are notoriously resistant to apoptotic stimuli, partly due to up-regulation of multiple anti-apoptotic genes by NF-κB transcription factor activation (Abu-Zant *et al.*, 2005; Losick and Isberg, 2006; Abu-Zant *et al.*, 2007), and possibly due to inhibition of the pro-apoptotic BH3-like proteins, BCL-RAMBO and BNIP3, by the *Legionella* effector protein SidF (Banga *et al.*, 2007). However, in the late stages of *Legionella* infection macrophages are typified by phenotypes linked to programmed apoptotic cell death, including nuclear condensation and activation of cysteine-dependent aspartic acid specific proteases (caspases) (Abu-Zant *et al.*, 2007; Santic *et al.*, 2007). This raises the possibility that *Legionella* may induce apoptosis in order to facilitate efficient egress. Recent studies, however, have shown that genetic deletion of the apoptotic executioner caspase-3, or of pro-apoptotic BCL-2 proteins, BAX and BAK, had little effect on overall *Legionella* burdens in cultured macrophages (Nogueira *et al.*, 2009), although host cell death rates were not investigated under these conditions.

A series of recent studies have documented that *Legionella* readily induce pyroptotic death, via nucleotide binding domain-leucine-rich repeat (NBD-LRR) NOD-like receptor 4 (NLRC4) inflammasome-mediated caspase-1 activation, in response to

detection of cytosolic bacterial flagellin by the adaptor protein NAIP5 (Ren *et al.*, 2006; Zhao *et al.*, 2011). While broth-derived *Legionella*, which express high levels of flagellin, fail to replicate intracellularly due to rapid pyroptotic host cell death, biofilm-derived *Legionella* down-regulate flagellin and evade pyroptosis (Abu Khweek *et al.*, 2013). Whether the up-regulation of flagellin synthesis during the final stages of *Legionella* replication promotes bacterial escape by triggering pyroptotic cell death, remains unknown (Byrne and Swanson, 1998; Molmeret *et al.*, 2010). In the absence of flagellin, *Legionella* may also induce pyroptosis via the non-canonical caspase-11 inflammasome, which senses cytosolic lipopolysaccharide (LPS) (Case *et al.*, 2013). While maintaining the integrity of the LCV membrane is integral to evading caspase-11 activation (Creasey and Isberg, 2011), it is not known whether cytosolic bacteria, which appear in the terminal phase of replication, subsequently trigger caspase-11-induced killing of macrophages.

The primary aim of the work described in this chapter was to investigate the role that the programmed cell death pathways, pyroptosis and apoptosis, as well as caspase-independent necroptosis, play during *Legionella* infection and egress. To do this, a novel imaging method was utilised that allowed for rigorous observation of *Legionella* infection of macrophages in real-time. Many conventional methods for following bacterial infection of host cells are limited because conclusions can be extrapolated from only a small number of isolated data points, meaning that subtle and important variations may be overlooked. However, by using fluorescent dyes and time-lapse microscopic imaging of infected cells, it was possible to quantify host cell death and mitochondrial integrity of individual primary bone marrow-derived macrophages (BMDMs) over the entire course of infection (up to 72 h). Ultimately, this proved to be

a powerful and sensitive technique for discerning the involvement of various host and bacterial cell death factors during *Legionella* infection.

3.2 Results

3.2.1 Wild-type and flagellin-deficient L. pneumophila induce different host cell death pathways

It is well established that the C57BL/6 mouse strain is restrictive for *L. pneumophila* infection. Intracellular flagellated wild-type (WT) *L. pneumophila*, derived from broth cultures or agar plates, triggers NAIP5/NLRC4/caspase-1-dependent pyroptotic cell death, which releases bacteria into the extracellular space. Caspase-1 also cleaves precursor Il-1β into its mature active form that, in the lungs, recruits activated neutrophils, resulting in bacterial clearance (Miao *et al.*, 2010). *In vitro*, released bacteria remain viable and are able to re-infect bystander macrophages to trigger repeated rounds of pyroptosis, but, subsequently, fail to replicate (Zamboni *et al.*, 2006). In contrast, *L. pneumophila* strains lacking flagellin (Δ*flaA*) are virulent in C57BL/6 mice and their derived macrophages because they do not activate NAIP5/NLRC4/caspase-1-dependent pyroptosis during the early stages of infection, thereby allowing the bacteria time to replicate before egressing to infect new cells.

Throughout the work described in this thesis, live-cell imaging of infected macrophages was employed to examine *Legionella* infection *in vitro* (Fig. 3.1). To do this, infected macrophages were labelled with fluorescent dyes, including: Cell Tracker Green (CTG) to identify and determine macrophage numbers; the cell-permeable orange-red fluorescent dye, tetramethylrhodamine methyl ester (TMRM), which is sequestered by active mitochondria and will fluoresce only while the outer mitochondrial membrane (OMM) remains intact; and Draq7, a fluorescent dye that only stains the nuclei of dead/permeabilised cells and is highly suitable for live-cell imaging due to its far-red spectrum.

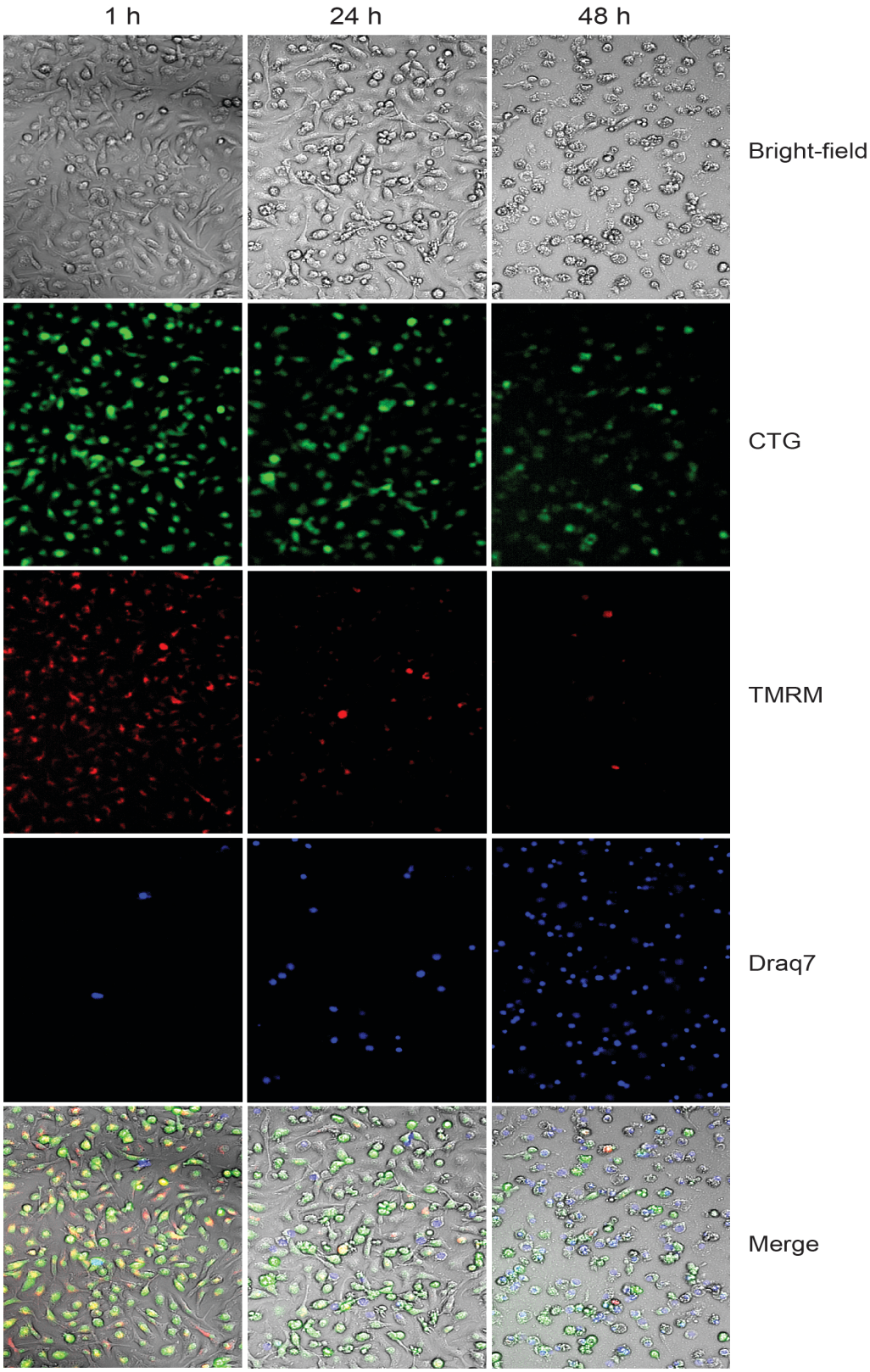


Figure 3.1 Live-cell imaging in *Legionella* infection. Live-cell imaging of wild-type (WT) C57BL/6 bone marrow-derived macrophages infected at an MOI of 10 with Δ*flaA L. pneumophila* and labelled with the fluorescent dyes Cell Tracker Green (CTG) (whole cells), tetramethylrhodamine methyl ester (TMRM) (active mitochondria), and Draq7 (dead cells) at 1, 24, and 48 h post-infection.

In order to first establish the sensitivity of using live-cell imaging to examine different modalities of host cell death during *Legionella* infection, the percentage of dead cells was quantified in Draq7-treated WT C57BL/6 BMDMs after infection with WT or Δ*flaA L. pneumophila*, at a multiplicity of infection (MOI) of 10 (Fig 3.2a). This MOI ensured that approximately 25 % of BMDMs were initially infected. Uninfected BMDMs were used to establish a baseline for Draq7 uptake. To determine that any host cell death induced by *Legionella* infection was effector-mediated, BMDMs were also infected with an avirulent *L. pneumophila* strain (Δ*dotA*) that, lacking a functional Type IV secretion system, fail to replicate or induce cell death under these conditions (Fig. 3.2b).

More than 90 % of WT BMDMs left uninfected, or infected with Δ*dotA L. pneumophila*, remained impermeable to Draq7 (*i.e.* healthy) after 72 h. In comparison, 80 – 90 % of WT BMDMs infected with WT or Δ*flaA L. pneumophila* were dead after 72 h. As expected, the WT BMDMs infected with WT *L. pneumophila* died more quickly than those infected with the Δ*flaA* strain; there was a lag of about 24 h between infection and the commencement of cell death in the Δ*flaA*-infected BMDMs, whereas approximately 50 % of WT-infected BMDMs were Draq7-positive by this time. This sigmoidal (*S*-shaped) death curve is characteristic of Δ*flaA L. pneumophila* infection and likely indicates repeated rounds of bacterial infection, replication, and egress.

Live-cell imaging was also used to quantify mitochondrial integrity over 72 h in BMDMs infected with WT, Δ*dotA*, and Δ*flaA L. pneumophila*, relative to uninfected BMDMs (Fig. 3.2c). In BMDMs infected with Δ*dotA L. pneumophila*, mitochondrial membrane potential (ΔΨm), which is indicative of intact mitochondria, was similar to

that of uninfected BMDMs, whereas in both WT- and Δ*flaA*-infected BMDMs, ΔΨm dropped by more that 50 % relative to uninfected BMDMs. Comparable to the Draq7 uptake in Fig. 3.2a, loss of ΔΨm occurred more quickly in the BMDMs infected with WT *L. pneumophila* than in those infected with Δ*flaA L. pneumophila*. These results clearly demonstrate that live-cell microscopic imaging, using Draq7 and TMRM fluorescent labelling to quantify cell death and mitochondrial integrity, respectively, is able to distinguish between the different cell death modalities and kinetics involved in *Legionella* infection.

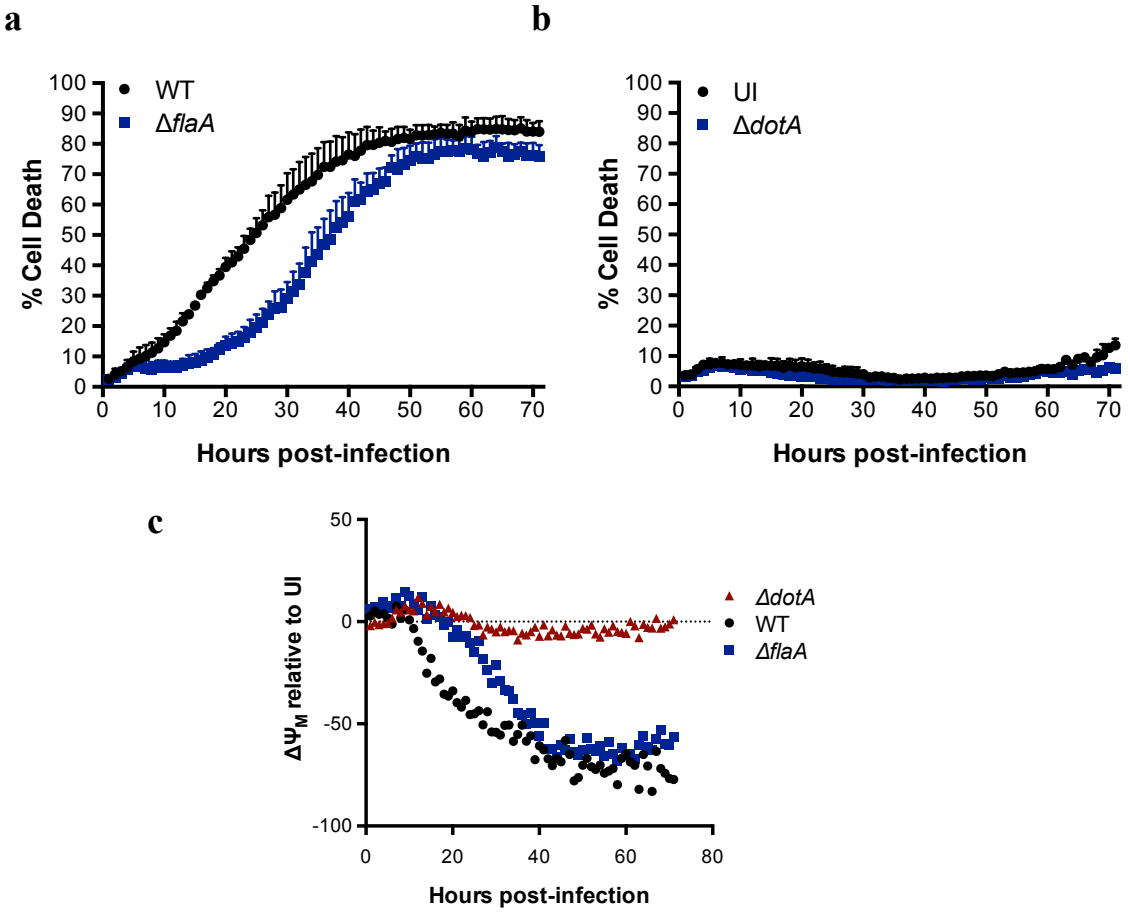


Figure 3.2 Wild-type and Δ*flaA L. pneumophila* induce different host cell death pathways a, b) Live-cell microscopic analysis showing **(a, b)** percentage cell death or **(c)** TMRM fluorescence (as a measure for mitochondrial integrity) over 72 h in Draq7-treated C57BL/6 WT BMDMs infected at a MOI of 10 with **(a, c)** WT *L. pneumophila* and Δ*flaA L. pneumophila*, or **(b)** Δ*dotA L. pneumophila*, and in BMDMs left uninfected (UI). TMRM fluorescence intensity is relative to that in uninfected BMDMs (dotted line). Data are representative of three independent experiments. Mean **(a, b, c)** and S.D. **(a, b)** of three independent biological replicates shown.

3.2.2 Only flagellin-deficient L. pneumophila can replicate in C57BL/6 BMDMs

To show that only Δ*flaA L. pneumophila* is able to replicate in WT C57BL/6-derived BMDMs, bacterial burdens [measured in colony-forming units (CFUs)] were calculated from BMDMs infected with either WT or Δ*flaA L. pneumophila* at 6, 24, and 48 h post-infection (Fig. 3.3). At 6 h post-infection there was no significant difference between the number of WT *L. pneumophila* and the number of Δ*flaA L. pneumophila* present in the macrophages. This is because bacterial replication only commences 6 – 8 h post-infection (Isberg *et al.*, 2009), suggesting that the initial infection level was similar. However, by 24 h, the total number of Δ*flaA* bacteria (derived from macrophages and culture supernatant) had increased approximately 10-fold, probably accounting for one round of infection and egress, whereas the number of WT bacteria remained static. At 48 h post-infection, the total number of Δ*flaA* bacteria had increased 300-fold, whilst the number of WT *L. pneumophila* did not increase significantly above the level at 6 h ($p > 0.05$). These results are in agreement with previous studies showing that flagellated *L. pneumophila* induces rapid macrophage cell death, which prevents replication of otherwise viable bacteria in C57BL/6-derived BMDMs. In contrast, *L. pneumophila* strains lacking flagellin are able to replicate under these conditions (Ren *et al.*, 2006).

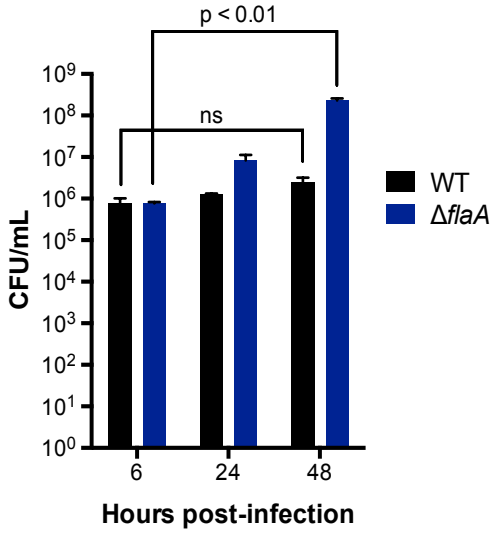


Figure 3.3 Only Δ*flaA L. pneumophila* can replicate in C57BL/6 BMDMs Bacterial burdens, shown in colony-forming units (CFU/mL), from wild-type (WT) and Δ*flaA L. pneumophila*-infected C57BL/6 WT BMDMs at 6, 24, and 48 h post-infection (MOI = 50, 2 h). Mean and S.E.M. of three independent experiments shown.

3.2.3 Legionella pneumophila does not require pyroptosis to egress from BMDMs

In order to examine a role for pyroptosis in the late-stages of infection as *Legionella* egress from spent macrophages, the ability of the Δ*flaA L. pneumophila* strain to induce pyroptotic cell death in caspase-1-deficient BMDMs, was tested. Because these mice also lack functional caspase-11 (Kayagaki *et al.*, 2011), BMDMs harvested from these mice are labelled as *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ double knock-outs. The health of WT C57BL/6 and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* at both a low MOI (5) and a high MOI (20) was monitored using live-cell imaging to quantify the percentage of dead (Draq7-positive) cells, over 72 h (Fig. 3.4a, b, c, d). Caspase-1 and caspase-11 are the only known executioners of pyroptotic cell death, and BMDMs deficient in both these proteins are resistant to all known pyroptotic stimuli (Bergsbaken *et al.*, 2009).

After 72 h of infection with Δ*flaA L. pneumophila*, approximately 70 – 80 % of the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs were Draq7-positive, irrespective of the MOI used, indicating that *L. pneumophila* is still able to induce cell death in the absence of both caspase-1 and caspase-11. As expected, there was very little observable cell death in the Δ*dotA*-infected BMDMs (Fig. 3.4b, d). Unexpectedly, however, cell death in the *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* at a MOI of 5 was delayed by close to 24 h compared to the WT BMDMs, although, by 72 h, the number of Draq7-positive cells was equivalent between the WT and caspase-1/11-deficient BMDMs. This is in contrast to cell death in the BMDMs infected at the higher MOI of 20, which was identical over the entire course of infection.

To test whether the delayed cell death in the caspase-1/11-deficient BMDMs affected bacterial numbers, bacterial burdens (CFU/mL) for the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs, were determined over the first 48 h of infection. Figure 3.4e shows that there was no significant difference between the total numbers of Δ*flaA* bacteria in the WT and in the *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs at 6, 24, and 48 h post-infection. In addition, CFU/mL were calculated from both the cell pellet (intracellular) and the supernatant (extracellular) 48 h after infection with Δ*flaA L. pneumophila* at a low MOI (Fig. 3.4f), as a measure for bacterial escape. However, despite there being approximately 30 % fewer Draq7-positive *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs at 48 h post-infection (Fig. 3.4a), there was no difference in bacterial numbers in the cell and supernatant fractions between the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs at this time-point. Taken together, these data demonstrate that *L. pneumophila* is able to induce flagellin-independent cell death in late-stage infections, which does not limit bacterial replication.

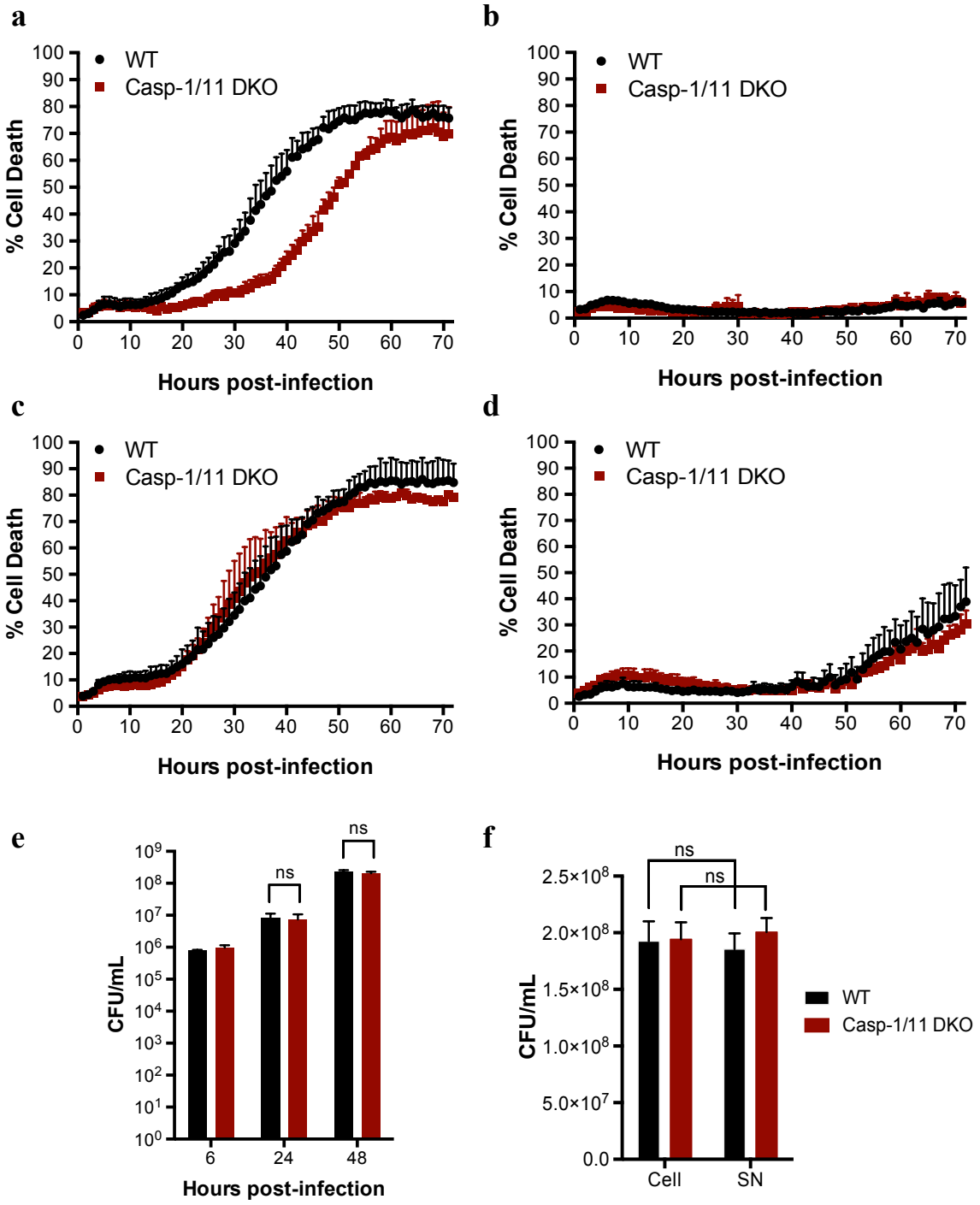


Figure 3.4 *Legionella pneumophila* induces pyroptosis in late-stage infection a, b, c, d) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT) and Caspase-1/11 double knock-out (DKO) BMDMs infected at a MOI of 5 with **(a)** Δ*flaA L. pneumophila* or **(b)** Δ*dotA L. pneumophila*, or at a MOI of 20 with **(c)** Δ*flaA L. pneumophila* or **(d)** Δ*dotA L. pneumophila.* Data are representative of two independent experiments. Mean and S.D. of three independent biological replicates shown. **e)** Bacterial burdens, (CFU/mL) from Δ*flaA L. pneumophila*-infected C57BL/6 WT and Caspase-1/11 DKO BMDMs at 6, 24, and 48 h post-infection (MOI = 5). Mean and S.E.M of three independent experiments shown. **f)** CFU/mL derived from cell and supernatant (SN) fractions of Δ*flaA L. pneumophila*-infected C57BL/6 WT and Caspase-1/11 DKO BMDMs at 48 h post-infection. Mean and S.E.M of two independent experiments shown.

3.2.4 Caspase-11-dependent cell death plays a role in Legionella infection under certain conditions

To determine whether the observed reduction in cell death rates in the *Caspase-1*$^{-/-}$ *Caspase-11*$^{-/-}$ BMDMs was due to caspase-1 or caspase-11, BMDMs that lacked only caspase-11 (*Caspase-11*$^{-/-}$) were infected with Δ*flaA L. pneumophila* at a MOI of 5 and 20 (Fig. 3.5a, b). Similar to the double knock-out BMDMs, the caspase-11-deficient BMDMs also showed a delay in cell death, compared to WT BMDMs, but only at the lower MOI. These data suggest that, under certain conditions, Δ*flaA L. pneumophila* activates a caspase-11-mediated cell death pathway independent of flagellin.

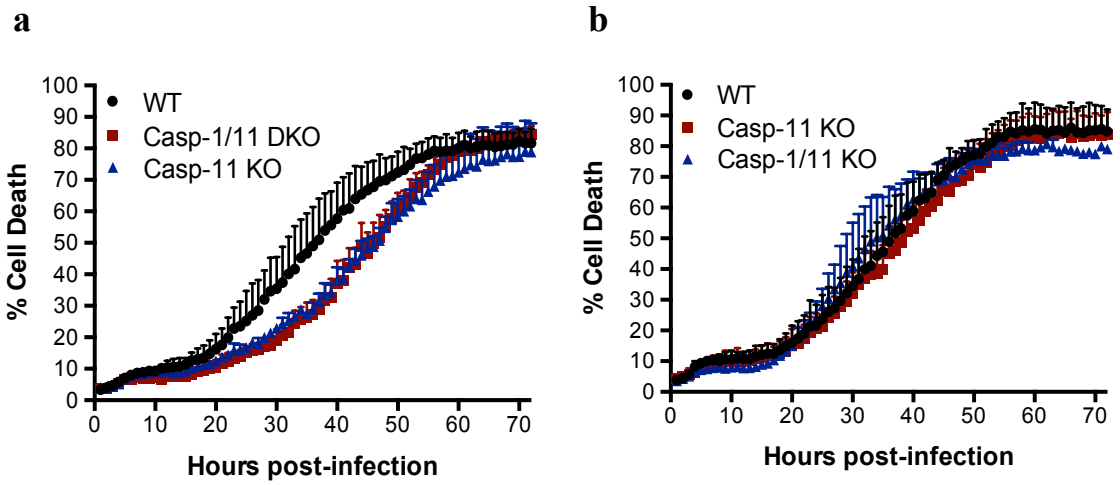


Figure 3.5 Caspase-11-dependent cell death plays a role in *Legionella* infection under certain conditions a, b) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT), Caspase-1/11 double knock-out (DKO), and Casp-11 KO BMDMs infected at a MOI of **(a)** 5 or **(b)** 20 with Δ*flaA L. pneumophila.* Data are representative of two independent experiments. Mean and S.D. of three independent biological replicates shown.

3.2.5 C57BL/6 macrophages restrict WT L. pneumophila infection independently of caspase-1

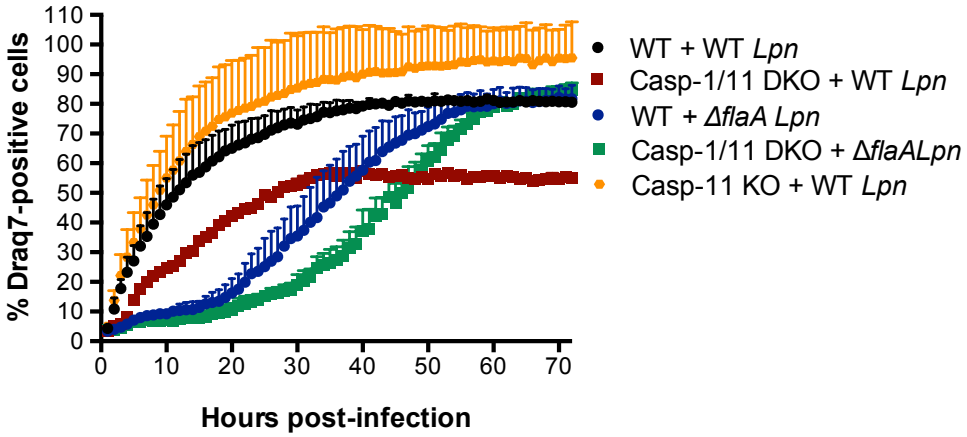
Given that *Legionella* are thought to induce flagellin expression in the terminal stage of infection, caspase-1/11-deficient BMDMs were also infected with flagellated WT *L. pneumophila* to test whether flagellin can induce other forms of cell death. Cell death

was compared over 72 h in WT C57BL/6, caspase-1/11-deficient and caspase-11-deficient BMDMs infected with WT and Δ*flaA L. pneumophila* (Fig. 3.6a). Without caspase-1 to initiate inflammatory cytokine processing and cell lysis in response to flagellin-sensing by the NLRC4 inflammasome complex, WT *L. pneumophila* should be able to replicate and induce cell death in a manner comparable to that of the Δ*flaA* strain. In the absence of caspase-1, cell death after WT *L. pneumophila* infection was markedly reduced, consistent with rapid cell death along the flagellin/NAIP5/NLRC4/caspase-1 pathway. Unexpectedly, however, the cell death induced by WT *L. pneumophila* in the caspase-1/11-deficient BMDMs was higher than Δ*flaA*-induced cell death in WT BMDMs, with approximately 50 % of WT *L. pneumophila* infected caspase-1/11-deficient BMDMs Draq7-positive at 24 h post-infection, compared to only 10 % of Δ*flaA*-infected WT BMDMs. Analysis of bright-field images also revealed differences in the cell death modalities (Fig. 3.6b). For example, WT *L. pneumophila* induced a highly lytic form of cell death in WT BMDMs, consistent with pyroptotic cell death, whereas the *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs showed increased blebbing and cell shrinkage, more akin to apoptosis. The latter also showed less Draq7 staining due to efferocytosis of apoptotic cells by bystander macrophages, thus accounting for the reduced cell death rates in the *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs, despite the majority of cells appearing dead by bright field imaging at 70 h. In contrast, WT *L. pneumophila* induced rapid cell death in both the WT and *Caspase-11*$^{-/-}$ BMDMs, suggesting that caspase-11 plays a minor role in WT *L. pneumophila* infections, likely due to rapid caspase-1 mediated host cell death.

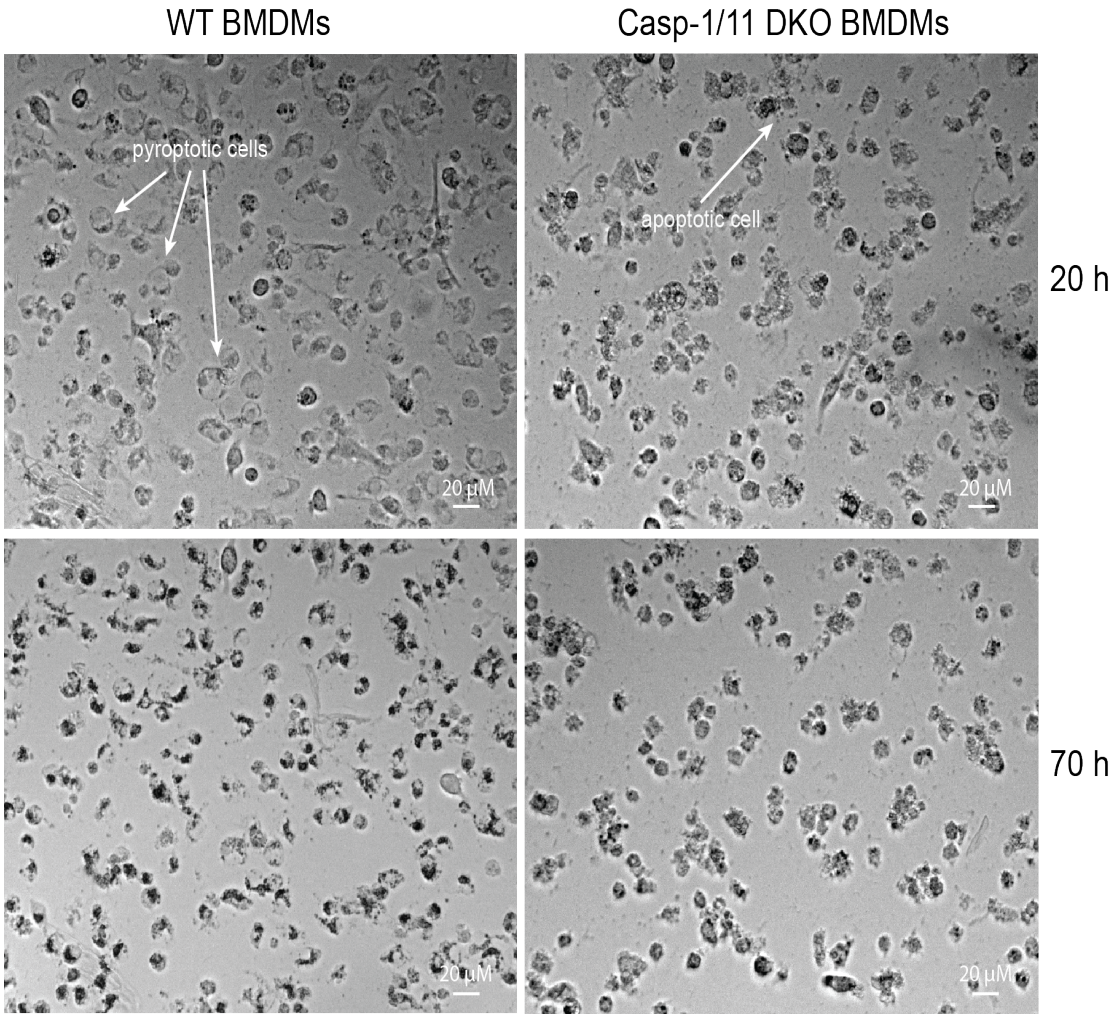
To examine whether there was a corresponding defect in *Legionella* replication, bacterial burdens were calculated in the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs

infected with WT and Δ*flaA L. pneumophila* over 48 h (Fig. 3.6c). Although WT *L. pneumophila* were able to replicate in the *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs compared with the WT BMDMs (10-fold difference), its replication was still impaired compared with that of the Δ*flaA* strain, which replicated over 100-fold in both cell types by 48 h. Together, these data suggest that *L. pneumophila* can induce both flagellin/caspase-1-dependent and flagellin-independent/caspase-11-dependent cell death (see Figure in Chapter 1), as well as an additional and, as yet uncharacterised, flagellin-dependent but caspase-1/11-independent form of cell death.

a


% Draq7-positive cells
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Hours post-infection
WT + WT *Lpn*
Casp-1/11 DKO + WT *Lpn*
WT + *ΔflaA Lpn*
Casp-1/11 DKO + Δ*flaALpn*
Casp-11 KO + WT *Lpn*

b


WT BMDMs
Casp-1/11 DKO BMDMs
pyroptotic cells
apoptotic cell
20 μM
20 h
70 h

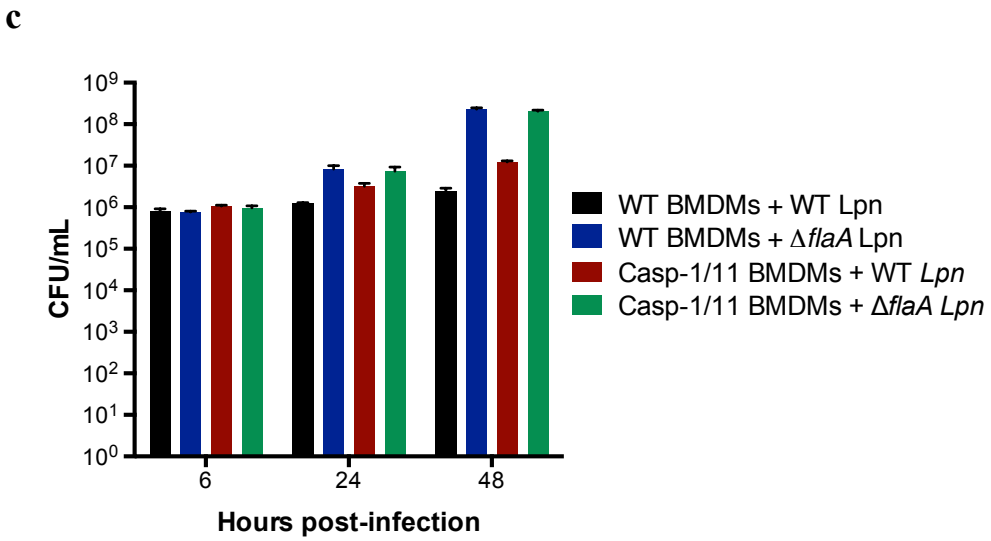


Figure 3.6 C57BL/6 macrophages restrict WT *L. pneumophila* infection independently of caspase-1 a) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT) and Caspase-1/11 DKO BMDMs infected at a MOI of 10 with WT *L. pneumophila*. Data are representative of two independent experiments. Mean and S.D. of three independent biological replicates shown. **b)** Bright-field images from WT and Casp-1/11 DKO BMDMs infected with Δ*flaA L. pneumophila* at a MOI of 10**,** taken at 20 and 70 h post-infection. Scale bar is 20 μM. **c)** Bacterial burdens (CFU/mL) from WT- and Δ*flaA*-infected C57BL/6 WT and Caspase-1/11 DKO BMDMs at 6, 24, and 48 h post-infection (MOI = 50, 2 h). Mean and S.E.M. of three independent experiments shown.

3.2.6 Permissive A/J-derived macrophages restrict WT L. pneumophila infection

To further validate the existence of a flagellin-dependent/caspase-1-independent cell death pathway in BMDMs, permissive A/J mouse-derived BMDMs were infected with WT and Δ*flaA L. pneumophila* (Fig. 3.7a). The A/J mouse strain has a 14 amino acid polymorphism in the autosomal recessive locus *lgn1* (Dietrich *et al.*, 1995), which maps to the gene for the adaptor protein NAIP5 (Yamamoto *et al.*, 1988; Diez *et al.*, 2003; Wright Jr *et al.*, 2003). Thus, A/J-derived BMDMs are deficient in flagellin-sensing, caspase-1-mediated pyroptosis and, therefore, are permissive for WT *L. pneumophila* replication.

As expected, there was very little death observed in the uninfected, or in the Δ*dotA*-infected, A/J BMDMs (Fig. 3.7b). In keeping with the notion that WT *L. pneumophila* infection is restricted independently of the NAIP5-NLRC4 axis, the A/J BMDMs infected with WT *L. pneumophila* died very quickly and were approximately 80 % Draq7-positive after only 24 h. This rapid cell death was similar to that observed with the C57BL/6 WT BMDMs infected with WT *L. pneumophila* (Fig. 3.2a), suggesting that these BMDMs are dying via a similar host-mediated mechanism. In comparison, the A/J BMDMs infected with the Δ*flaA* strain showed the classic *S*-shaped death curve associated with repeated rounds of *Legionella* infection, replication, and egress (as seen in Fig. 3.2a). Thus, A/J-derived BMDMs are not permissive for all *L. pneumophila* strains and are able to restrict flagellated WT *L. pneumophila* infection, likely independently of the NLRC4/caspase-1 axis.

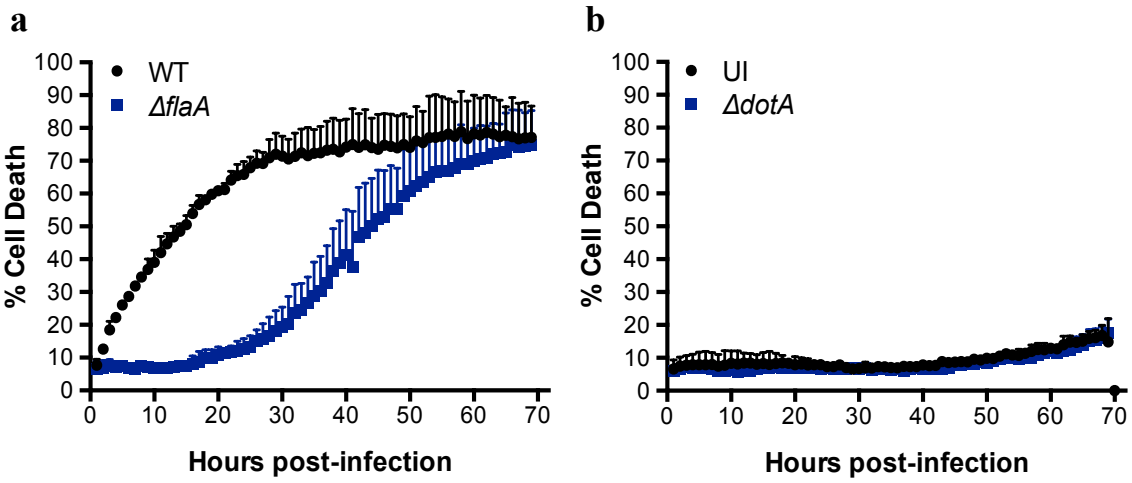


Figure 3.7 A/J-derived macrophages sense flagellated *L. pneumophila* a, b) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated A/J mouse-derived BMDMs infected at a MOI of 10 with **(a)** wild-type (WT) and Δ*flaA L. pneumophila*, or **(b)** Δ*dotA L. pneumophila*, or in BMDMs left uninfected (UI). Mean and S.D. of three independent biological replicates shown. Data are representative of three independent experiments.

3.2.7 WT L. longbeachae infection is not restricted in C57BL/6 macrophages

Next, the health of WT C57BL/6 and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs infected with WT *L. longbeachae* was determined in order to further investigate the existence of the non-canonical flagellin-dependent/caspase-1/11-independent host cell death pathway (Fig. 3.8a). Unlike WT *L. pneumophila*, *L. longbeachae* does not activate NLRC4/caspase-1-mediated pyroptotic cell death (Asare *et al.*, 2007) because it does not carry the necessary flagellar biosynthesis genes (Cazalet *et al.*, 2010).

Treatment of WT BMDMs with heat-killed (HK) *L. longbeachae* did not cause cell death (Fig. 3.8b). In contrast, live *L. longbeachae* caused rapid cell death in both the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs, but only after a lag phase of 24 h, reminiscent of killing induced by the Δ*flaA L. pneumophila* strain (Fig. 3.2a). There was no significant difference between the rate of killing in the WT and the *Caspase-1*$^{-/-}$ *Caspase-11*$^{-/-}$ BMDMs. Similar to Δ*flaA L. pneumophila*, WT *L. longbeachae* also showed robust growth in both the WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs at 48 h post-infection (Fig 3.8c). Taken together, these results demonstrate that: (1) *L. longbeachae* replicates and induces cell death in BMDMs independently of both caspase-1 and caspase-11; and (2) *L. longbeachae* infection is not restricted via a similar caspase-1/11-independent mechanism to that of WT *L. pneumophila*, due to its lack of a flagellum. This suggests, that *L. longbeachae*, but also *L. pneumophila,* induce other forms of macrophage cell death in the late stages of infection.

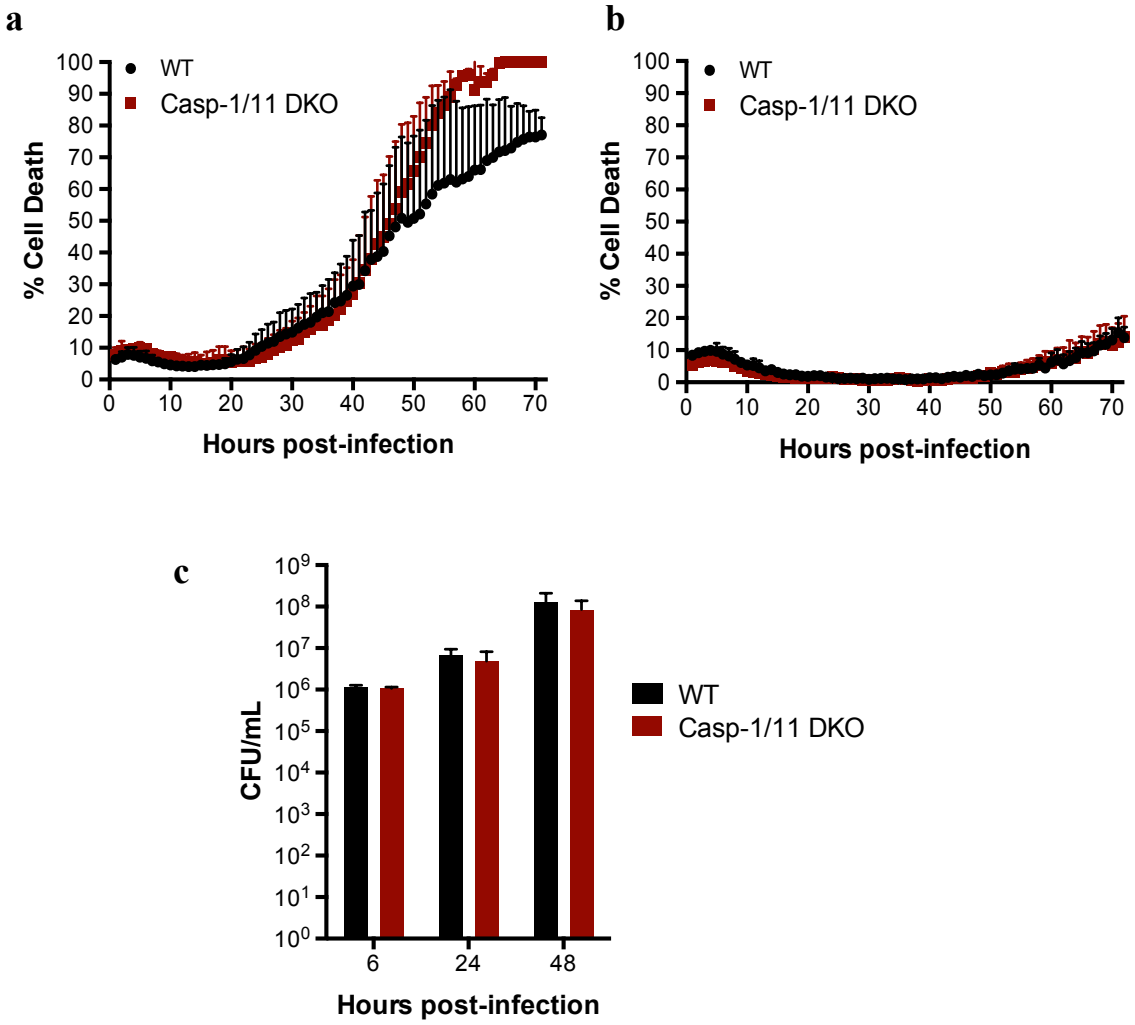


Figure 3.8 WT *L. longbeachae* infection is not restricted in C57BL/6 macrophages a, b) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT) and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs infected at a MOI of 10 with **(a)** WT *L. longbeachae*, or **(b)** heat-killed (HK) *L. longbeachae.* Data are representative of three independent experiments. Mean and S.D. of three independent biological repeats shown. **c)** Bacterial burdens, (CFU/mL) from WT *L. longbeachae*-infected WT and *Caspase-1*$^{-/-}$*Caspase-11*$^{-/-}$ BMDMs at 6, 24, and 48 h post-infection (MOI = 50, 2 h). Mean and S.E.M of three independent experiments shown.

3.2.8 Legionella pneumophila infection does not require BAX or BAK

Besides pyroptosis, several studies have suggested that *Legionella* induces mitochondrial apoptosis in the late stages of infection (Molmeret *et al.*, 2004a; Abu-Zant *et al.*, 2005). BAX and BAK are essential for induction of canonical mitochondrial apoptosis (Ola *et al.*, 2011). Therefore, to test whether apoptotic cell death contributes to *Legionella* replication, or its ability to induce cell death, BMDMs deficient in BAK, and in both BAX and BAK, were infected with Δ*flaA L. pneumophila*.

As expected, in the absence of BAK, or BAX and BAK together, both uninfected and Δ*dotA L. pneumophila*-infected BMDMs remained viable (Fig. 3.9a and b), indicating that neither BAX nor BAK were essential to cell survival under these conditions. Both the $BAK^{-/-}$ and the $BAX^{-/-}BAK^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* underwent cell death at a similar rate to the Δ*flaA*-infected WT BMDMs (Fig. 3.9c). Although the deletion of both BAX and BAK resulted in a 15 – 20 % decrease in the rate of cell death compared to WT, or deletion of BAK alone, this was not significant and, by 72 h, the total number of Draq7-positive BMDMs was comparable between all three macrophage lines.

Comparison of bacterial burdens (CFU/mL) from WT, $BAK^{-/-}$, and $BAX^{-/-}BAK^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* showed that there was no significant difference between the CFU/mL in the WT, $BAK^{-/-}$, or $BAX^{-/-}BAK^{-/-}$ BMDMs after 48 h of infection with Δ*flaA L. pneumophila* ($p > 0.05$) (Fig. 3.9d). Together, these data suggest that BAX/BAK-mediated apoptosis is not required for bacterial replication, nor is it the primary pathway for inducing host cell death during egress.

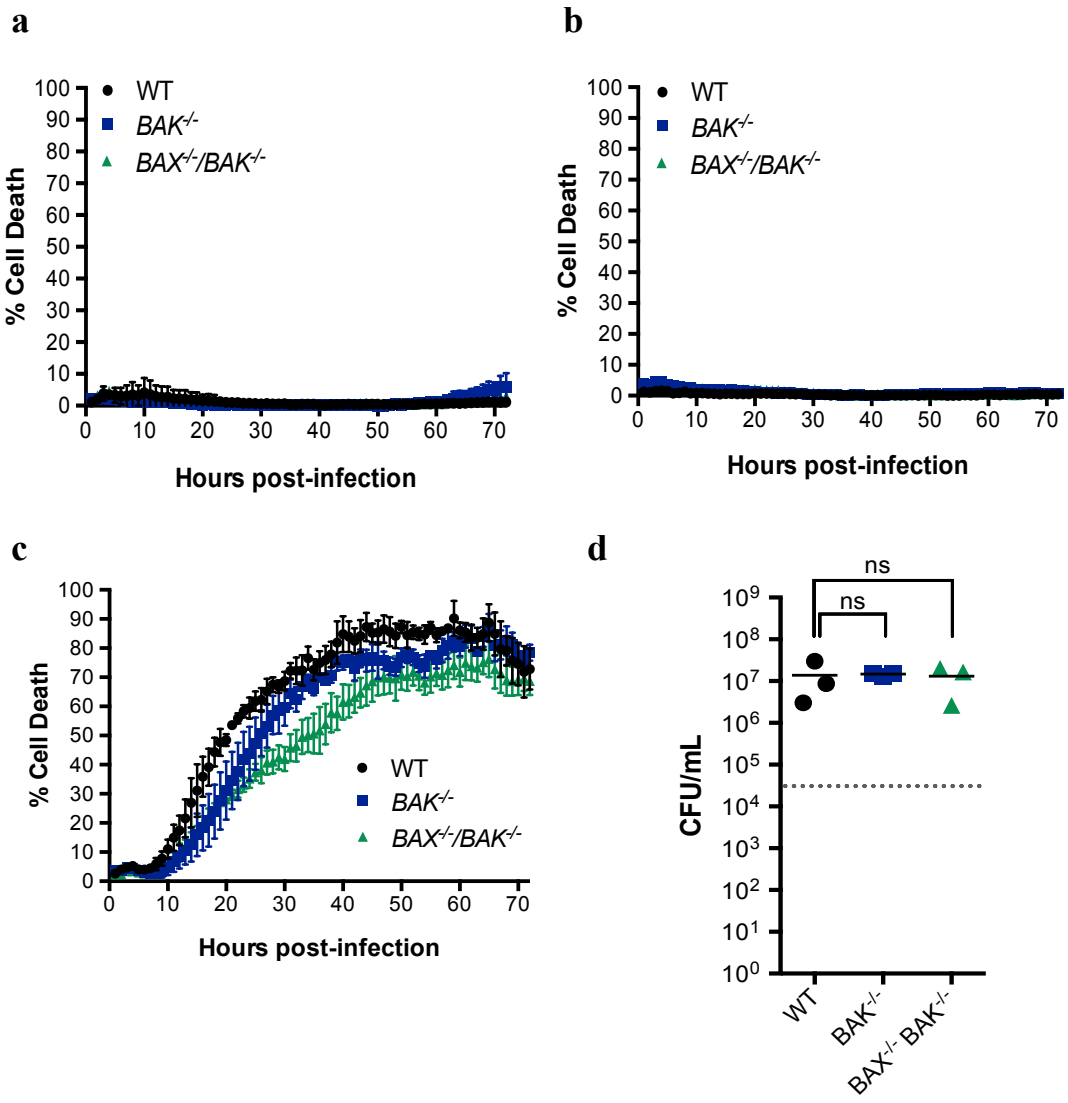


Figure 3.9 *L. pneumophila* infection does not require BAX or BAK a, b, c) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT), *BAK*$^{-/-}$ and *BAX*$^{-/-}$*BAK*$^{-/-}$ BMDMs infected at a MOI of 10 with **(a)** Δ*dotA L. pneumophila*, **(c)** Δ*flaA L. pneumophila*, or **(b)** in uninfected BMDMs. Data are representative of three independent experiments. Mean and S.D. of three biological replicates shown. **(d)** Bacterial burdens (CFU/mL) calculated from C57BL/6 wild-type (WT), *BAK*$^{-/-}$, and *BAX*$^{-/-}$*BAK*$^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* for 48 h (MOI = 50, 2 h). The dotted line shows the CFU/mL at 6 h post-infection Mean of three independent experiments shown.

3.2.9 Treatment of BMDMs with a pan-caspase inhibitor (Q-VD-OPh) does not impede Legionella infection or egress

Legionella export at least six effector molecules that target mitochondria and several of these have been shown to induce cytochrome-*c* release and apoptosis, possibly independent of BAX/BAK (Zhu *et al.*, 2013). To test whether any of the apoptotic caspases contribute to the ability of *Legionella* to induce cell death in BMDMs, Δ*flaA*-infected BMDMs were treated with pan-caspase inhibitor Q-VD-OPh (Q-VD), which effectively blocks cyclohexamide-induced apoptotic cell death in BMDMs (Fig. 3.10).

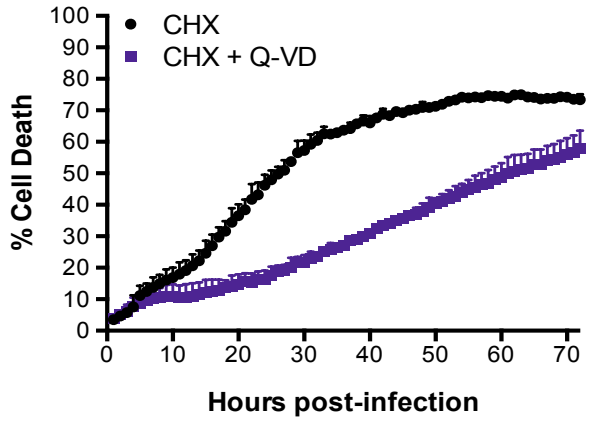


Figure 3.10 Treatment with Q-VD rescues cells from cycloheximide-induced apoptosis Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT) BMDMs treated with 1 μg.mL^{-1} cycloheximide (CHX) with, or without, 20 μM of Q-VD. Data are representative of two independent experiments. Mean and S.D. of three independent biological replicates shown.

Pre-treatment with Q-VD did not induce cell death in the uninfected, Δ*dotA*-infected, or HK *L. longbeachae*-infected BMDMs, even after 72 h (Fig. 3.11a and b). Meanwhile, the addition of Q-VD before infection did not prevent either Δ*flaA L. pneumophila* or WT *L. longbeachae* from inducing cell death in macrophages (Fig. 3.11c and d). Both the rate at which *L. pneumophila* and *L. longbeachae* were able to

induce cell death, as well as the total number of BMDMs that were Draq7-positive after 72 h (~80 %), were the same between Q-VD-treated and untreated cells.

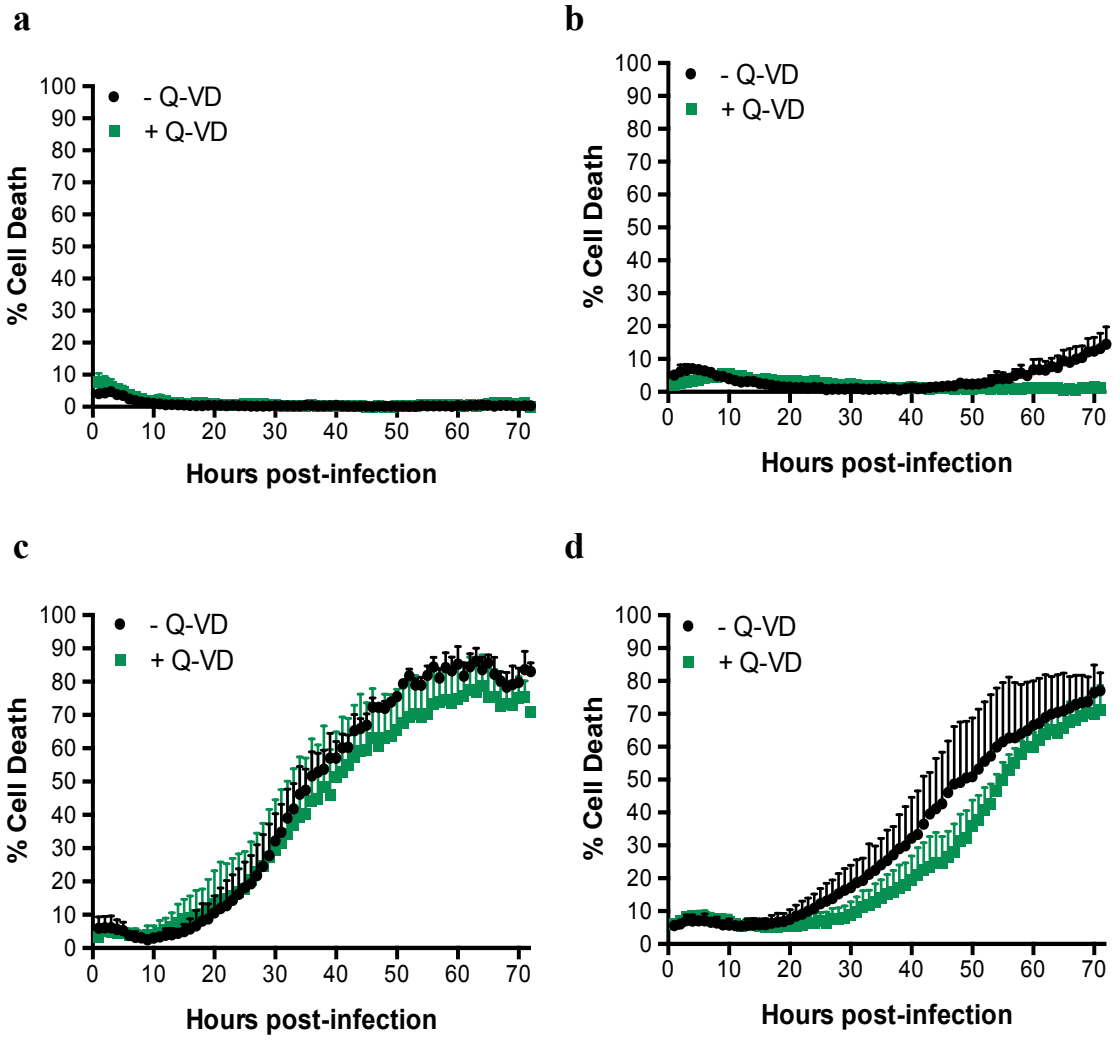


Figure 3.11 Treatment with Q-VD does not impede *Legionella* infection or egress a, b, c) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT) BMDMs infected at a MOI of 10 with **(a)** Δ*dotA L. pneumophila*, **(b)** heat-killed (HK) *L. longbeachae* **(c)** Δ*flaA L. pneumophila*, or **(d)** WT *L. longbeachae*, following treatment with 20 μM Q-VD. Data are representative of three independent experiments. Mean and S.D. of three independent biological replicates shown.

To investigate whether *Legionella* were still able to replicate normally in BMDMs pre-treated with Q-VD, bacterial burdens were calculated at 48 h post-infection from WT BMDMs pre-treated with Q-VD and infected with Δ*flaA L. pneumophila* (Fig. 3.12a)

or WT *L. longbeachae* (Fig. 3.12b). In keeping with the live-cell imaging data, after 48 h there was no significant difference in total bacterial numbers recovered from BMDMs treated with Q-VD, or left untreated, for either Δ*flaA L. pneumophila* or WT *L. longbeachae* ($p > 0.05$). Taken altogether, these results do not support a major role for canonical mitochondria-mediated apoptotic cell death during *Legionella* infection.

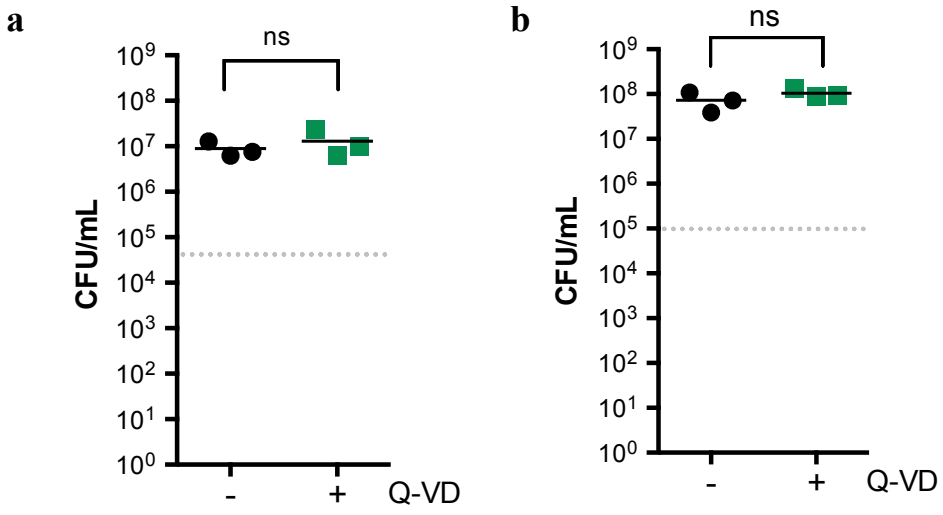


Figure 3.12 Treatment with Q-VD does not impede *Legionella* replication a, b) Bacterial burdens (CFU/mL) calculated from C57BL/6 wild-type (WT) BMDMs pre-treated with the 20 μM Q-VD and infected at with **(a)** Δ*flaA L. pneumophila*, or **(b)** WT *L. longbeachae* for 48 h (MOI = 50, 2 h). The dotted line shows the CFU/mL at 6 h post-infection Mean of three independent experiments shown.

3.2.10 RIPK3, Caspase-8, and MLKL are not required for in vitro Legionella infection

Macrophages are also able to induce caspase-independent cell death, termed necroptosis. Necroptosis is a form of regulated necrosis dependent upon the activity of receptor-interacting protein kinase-1 (RIPK1) and/or RIPK3 (Holler *et al.*, 2000; Newton, 2015). Binding of extracellular ligands to cell death receptors, *e.g.*, TNFR1, assembles a death-induced signalling complex (DISC) that includes RIPK1 and RIPK3 (Vanden Berghe *et al.*, 2014). In the absence of caspase-8, which blocks RIPK3

signalling, active RIPK3 recruits and phosphorylates the mixed lineage kinase domain-like (MLKL) protein, thereby disrupting plasma membrane integrity to trigger necrotic cell death. Again, mitochondrial dysfunction has been implicated as having a critical role in this process, although this has recently been challenged [reviewed in Marshall and Baines, (2014)].

To test whether there was any role for necroptosis during *Legionella* infection or egress, WT C57BL/6, *RIPK3*$^{-/-}$, *RIPK3*$^{-/-}$*caspase-8*$^{-/-}$, and *MLKL*$^{-/-}$ BMDMs were infected with Δ*flaA L. pneumophila* (Fig. 3.13a) or WT *L. longbeachae* (Fig. 3.13b). The RIPK3/Caspase-8 double knock-out was used because caspase-8 deletion alone is embryonic lethal (Varfolomeev *et al.*, 1998), and this phenotype is ameliorated by RIPK3 co-deletion (Kaiser *et al.*, 2011). As expected, more than 90 % of BMDMs infected with Δ*dotA L. pneumophila* or HK *L. longbeachae* remained Draq7-negative after 72 h (Fig. 3.13c and d). In contrast, the WT, *RIPK3*$^{-/-}$, *RIPK3*$^{-/-}$*caspase-8*$^{-/-}$, and *MLKL*$^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* all showed the classic *S*-shaped death curve associated with repeated rounds of *Legionella* infection, replication, and egress. Although the *L. longbeachae* induced cell death more quickly than the Δ*flaA* strain, there was no difference between the rate and extent of cell death in the knock-out BMDMs versus the WT BMDMs, for either *Legionella* species.

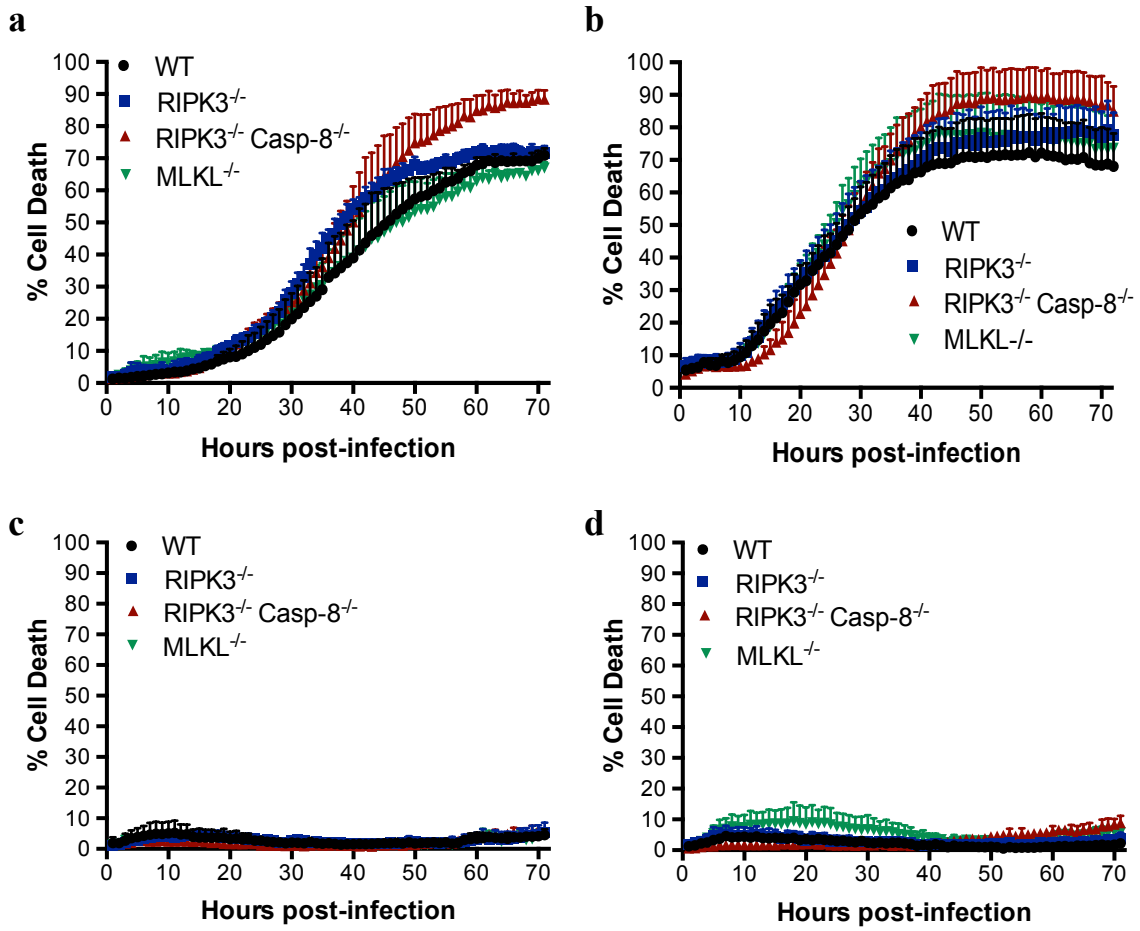


Figure 3.13 RIPK3, Caspase-8, and MLKL are not required for *in vitro* *Legionella* infection a, b, c, d) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 wild-type (WT), RIPK3$^{-/-}$, RIPK3$^{-/-}$/Caspase-8$^{-/-}$, and MLKL$^{-/-}$ BMDMs infected at a MOI of 10 with **(a)** Δ*flaA L. pneumophila*, **(b)** WT *L. longbeachae,* **(c)** Δ*dotA L. pneumophila*, or **(d)** heat-killed (HK) *L. longbeachae*. Mean and S.D. shown. Data are representative of three independent experiments.

To confirm that *L. pneumophila* and *L. longbeachae* were able to replicate normally within BMDMs deficient in these necroptotic factors, bacterial burdens were calculated at 48 h post-infection from WT, RIPK3$^{-/-}$, RIPK3$^{-/-}$/Caspase-8$^{-/-}$, and MLKL$^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* (Fig. 3.14a), or WT *L. longbeachae* (Fig. 3.14b). After 48 h, there was no significant difference in either Δ*flaA L. pneumophila* or *L. longbeachae* numbers between the WT, RIPK3$^{-/-}$, RIPK3$^{-/-}$

/Caspase-8$^{-/-}$, and MLKL$^{-/-}$ BMDMs; both *L. pneumophila and L. longbeachae* had replicated approximately 100-fold in all BMDMs over this period. Together, these data indicate that *Legionella* does not require the essential necroptotic cell death factors, RIPK3 and MLKL, nor apoptotic caspase-8 to replicate or induce cell death in macrophages.

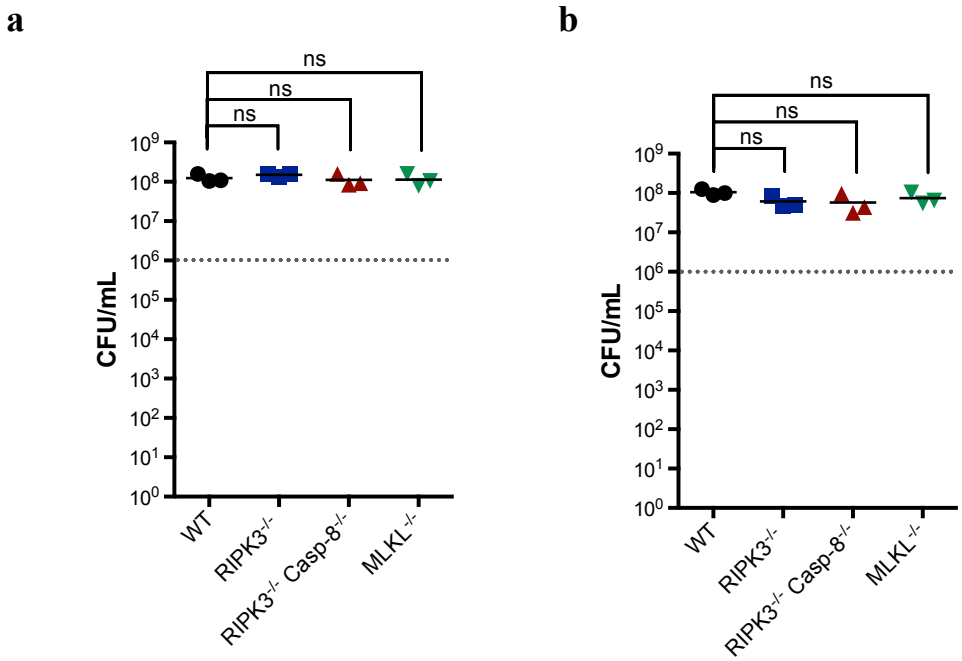


Figure 3.14 RIPK3, Caspase-8, and MLKL are not required for *in vitro* *Legionella* replication a, b) Bacterial burdens (CFU/mL) calculated from C57BL/6 wild-type (WT), RIPK3$^{-/-}$, RIPK3$^{-/-}$/Caspase-8$^{-/-}$, and MLKL$^{-/-}$ BMDMs infected with **(a)** Δ*flaA L. pneumophila*, or **(b)** WT *L. longbeachae* for 48 h (MOI = 50, 2 h). The dotted line shows the CFU/mL at 6 h post-infection Mean from three independent experiments shown.

3.2.11 RIPK3, Caspase-8, and MLKL are not required for in vivo Legionella infection

To test if the ability to induce necroptotic cell death is also not important for *Legionell*a infection *in vivo*, bacterial burdens were calculated from the lungs of WT C57BL/6 mice, and mice lacking RIPK3, RIPK3/Caspase-8, or MLKL after 72 h of infection with *L. longbeachae* (Fig. 3.15). In keeping with the *in vitro* data, there was

no significant difference in bacterial numbers between the different mouse knock-out strains after 72 h of infection with *L. longbeachae* ($p > 0.05$).

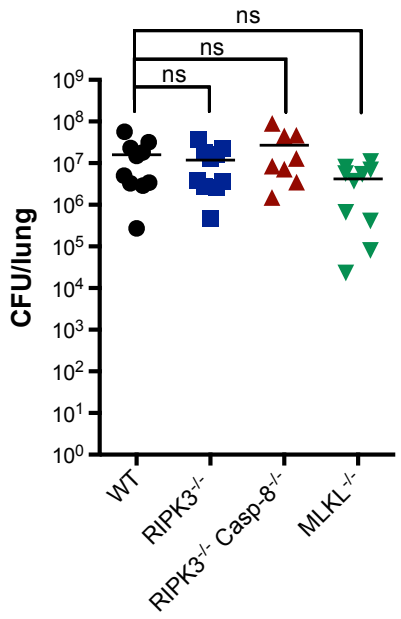


Figure 3.15 RIPK3, Caspase-8, and MLKL are not required for *in vivo Legionella* infection Bacterial burdens (CFU/lung) collected from the lungs of C57BL/6 wild-type (WT), RIPK3$^{-/-}$, RIPK3$^{-/-}$/Caspase-8$^{-/-}$, and MLKL$^{-/-}$ mice, 72 h after infection with 1×10^5 WT *L. longbeachae*. Mean shown. Each data point represents one mouse.

3.2.12 Legionella pneumophila induces cell death independently of Cyclophilin-D

Mitochondria may also induce cell death via the activation of a mitochondrial permeability transition pore (mPT), which is induced by Ca^{2+} release or by oxidative damage. The *Ppif* gene product, Cyclophilin-D, is a critical component of the mPT that functions independently of the apoptotic BCL-2 network (Baines *et al.*, 2005). To test whether Cyclophilin-D had any involvement in induction of cell death during *Legionella* infection, WT C57BL/6 and *Ppif*$^{-/-}$ BMDMs were infected with Δ*flaA L. pneumophila* (Fig. 3.16).

As expected the uninfected and the Δ*dotA*-infected BMDMs were all impermeable to

Draq7 for the first 24 h (Fig. 3.16a and b), and the avirulent Δ*dotA* strain did not induce increased cell death over 48 h of infection, compared with uninfected BMDMs. In contrast, there was substantially more cell death apparent in the Δ*flaA*-infected BMDMs, which were 70 – 80 % Draq7-positive after 48 h, and both the WT and *Ppif*$^{-/-}$ BMDMs showed classic S-shaped death curves associated with repeated rounds of *Legionella* infection, replication, and egress (Fig 3.16c). This result demonstrates that *Legionella* can still induce cell death normally in the absence of a functional mitochondrial permeability transition pore complex.

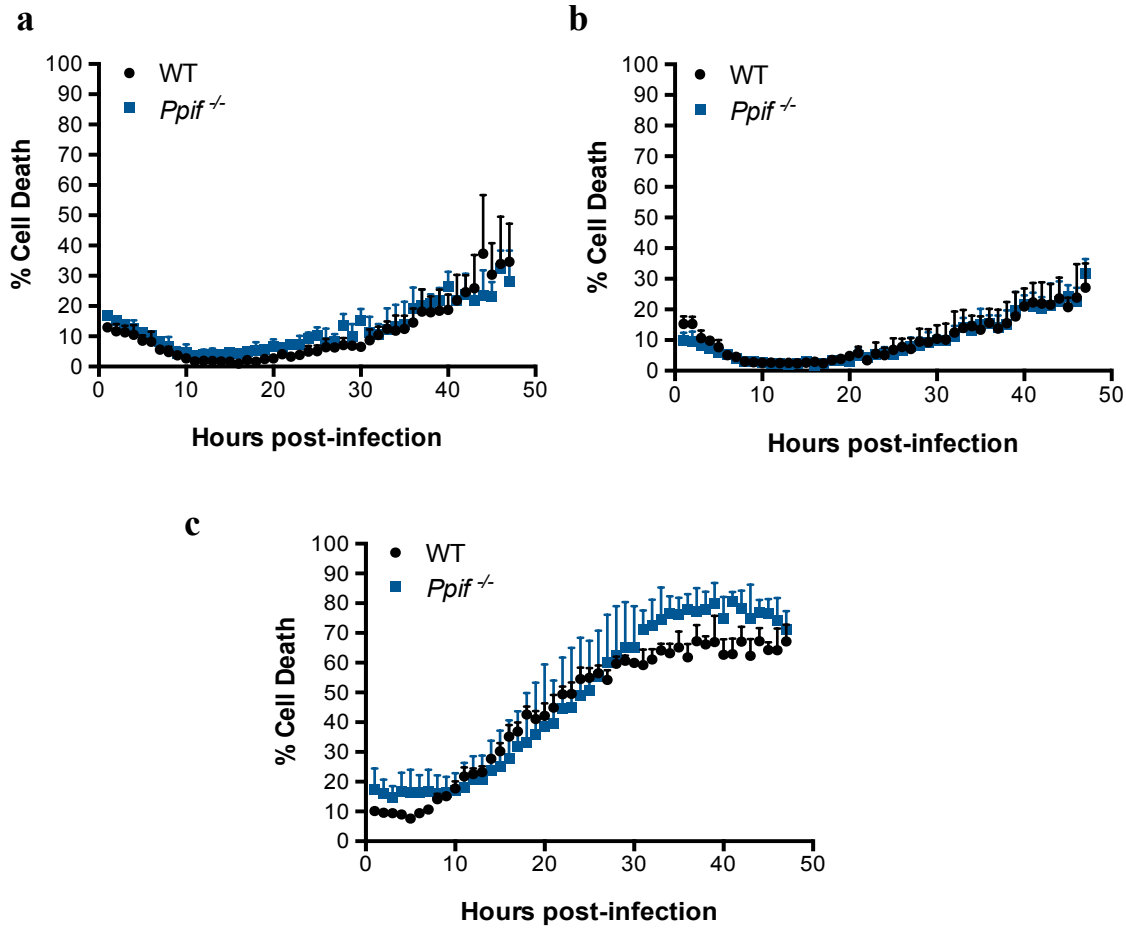


Figure 3.16 *Legionella pneumophila* induces cell death independent of Cyclophilin-D a, b, c) Live-cell microscopic analysis showing percentage cell death over 48 h in Draq7-treated C57BL/6 wild-type (WT) and *Ppif*$^{-/-}$ BMDMs infected at a MOI of 10 with **(a)** Δ*dotA L. pneumophila*, **(c)** Δ*flaA l. pneumophila*, or **(b)** uninfected BMDMs. Data are representative of three independent experiments. Mean and S.D. of three independent biological replicates shown.

3.2.13 Loss of L. pneumophila effector SidF does not induce increased apoptotic cell death of infected BMDMs.

The *L. pneumophila* effector protein SidF is the only effector known to target and inhibit host cell apoptotic factors. Previous published results using TUNEL staining have shown that, in the absence of SidF, an increased number of BMDMs undergo apoptosis during the early stages of infection (Banga *et al.*, 2007). Therefore, in order to confirm that SidF deletion resulted in an increase in host cell apoptotic death, the health of WT C57BL/6 BMDMs over 72 h was quantified following infection with the *L. pneumophila* strains Δ*dotA*, Δ*flaA*, Δ*flaA*/Δ*sidF*, and the complemented Δ*flaA*/Δ*sidF* strain (+ SidF) using live-cell imaging (Fig. 3.17a). As expected, the Δ*dotA*-infected BMDMs remained impermeable to Draq7 after 72 h. Surprisingly, there was no difference between the rate or extent of cell death induced by the Δ*flaA*, the Δ*flaA*/Δ*sidF*, or the Δ*flaA*/Δ*sidF* + SidF strains. Furthermore, less than 2 % of the Δ*flaA*/Δ*sidF*-infected BMDMs underwent apoptotic cell death during the first 48 h of infection, similar to the number of Δ*flaA*-infected BMDMs (Fig. 3.17b). Finally, consistent with this finding, there was no detectable caspase-3 activation in either the Δ*flaA*- or Δ*flaA*/Δ*sidF*-infected BMDMs after 8, 12, and 24 h of infection, as measured by immunoblotting of the active caspase-3 p17/p19 fragment (Fig. 3.17c).

Mitochondrial permeabilisation during apoptosis occurs before cells become permeable to Draq7. Therefore, in order to better determine if cells infected with the Δ*flaA*/Δ*sidF L. pneumophila* strain were undergoing apoptosis during the early stages of infection, mitochondrial integrity was also quantified over 72 h in BMDMS infected with Δ*dotA*, Δ*flaA*, and Δ*flaA*/Δ*sidF*, relative to uninfected BMDMs (Fig. 3.17d). As expected, mitochondria from BMDMs infected with the Δ*dot*A strain remained intact after 72 h. In contrast, and analogous to the data obtained with Draq7, there was no

difference in the rate or extent of mitochondrial outer membrane permeabilisation between BMDMs infected with the Δ*flaA* or Δ*flaA*/Δ*sidF L. pneumophila*, suggesting that loss of SidF does not result in increased apoptosis of infected macrophages at any stage of infection

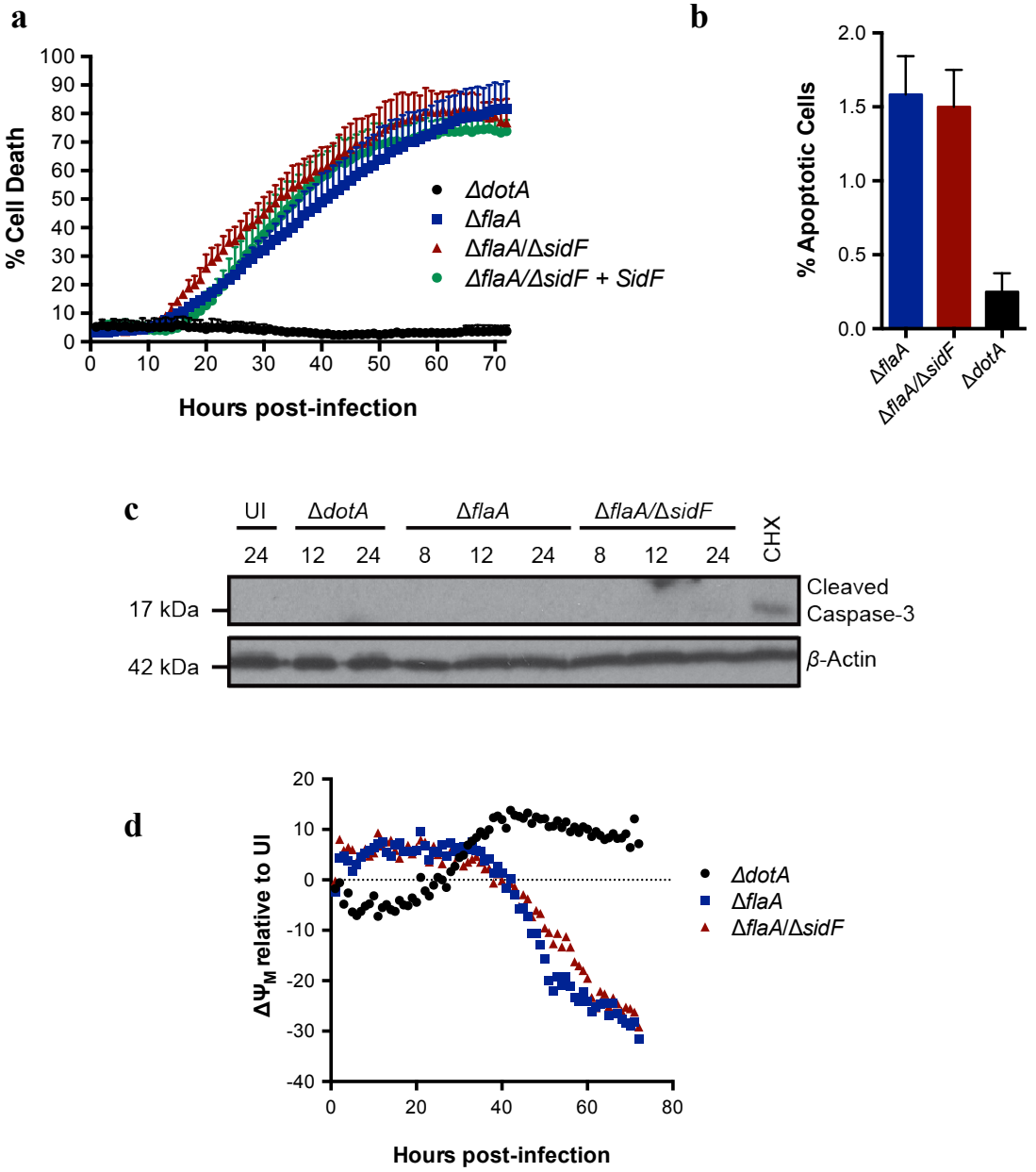


Figure 3.17 Loss of SidF does not induce apoptotic cell death of infected BMDMs a) Live-cell microscopic analysis showing percentage cell death over 72 h in Draq7-treated C57BL/6 BMDMs infected at a MOI of 10 with the *L. pneumophila* strains Δ*dotA*, Δ*flaA,* Δ*flaA*/Δ*sidF,* and Δ*flaA*/Δ*sidF* + SidF. Data are representative of three independent experiments. Mean and S.D. of three independent biological replicates shown. **b)** Percentage apoptotic cells, determined by membrane blebbing, over 48 h in BMDMs infected with the *L. pneumophila* strains Δ*dotA*, Δ*flaA*, and Δ*flaA*/Δ*sidF* at an MOI of 20. Mean and S.D. shown. > 800 cells scored. **c)** Time course immuno-blot analysis for cleaved (*i.e.*, active) caspase-3 in BMDMs infected with Δ*flaA* or Δ*flaA*/Δ*sidF L. pneumophila.* BMDMs treated with 10 μM cycloheximide (CHX) were used as a positive control. Actin blot is a loading control. **d)** Live-cell microscopic analysis over 72 h showing TMRM fluorescence intensity (as a measure for mitochondrial integrity) in C57BL/6 BMDMs infected at a MOI of 10 with Δ*dotA*, Δ*flaA*, or Δ*flaA*/Δ*sidF L. pneumophila.* Data shown relative to TMRM fluorescence intensity in uninfected BMDMs (dotted line). Mean from three independent experiments shown.

In order to determine whether SidF deletion had any effect on *L. pneumophila* replication in BMDMs, as previously reported (Banga *et al.*, 2007), bacterial burdens (CFU/mL) from BMDMs infected with Δ*flaA*, Δ*flaA*/Δ*sidF*, or Δ*flaA*/Δ*sidF* + SidF *L. pneumophila* were compared at 48 h post-infection (Fig. 3.18). The data showed that, after 48 h of infection, there was a small but significant difference (≃3-fold) between the CFU/mL recovered with the Δ*flaA*/Δ*sidF* strain, compared to the Δ*flaA* or Δ*flaA*/Δ*sidF* + SidF *L. pneumophila* strains ($p < 0.01$).

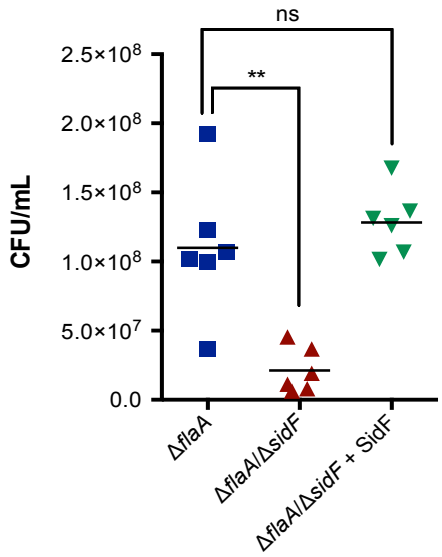


Figure 3.18 Loss of SidF moderately impedes *L. pneumophila* replication *in vitro* Bacterial burdens (CFU/mL) calculated from C57BL/6 BMDMs infected with the *L. pneumophila* strains Δ*flaA*, Δ*flaA/*Δ*sidF,* and Δ*flaA/*Δ*sidF* + SidF for 48 h (MOI = 50, 2 h). Mean of three independent experiments shown. ** = $p < 0.01$.

3.2.14 The effector SidF is not required for L. pneumophila infection in vivo

Given the observed, albeit marginal, growth defect of the Δ*flaA/*Δ*sidF* strain in BMDMs, the role of SidF was examined in *L. pneumophila* infection *in vivo*. For this, bacterial burdens were calculated from the lungs of WT C57BL/6 mice 48 h after infection with either Δ*flaA* or Δ*flaA*/Δ*sidF L. pneumophila* (Fig. 3.19). The data

showed that there was no significant difference in bacterial numbers between the two *L. pneumophila* strains ($p > 0.05$).

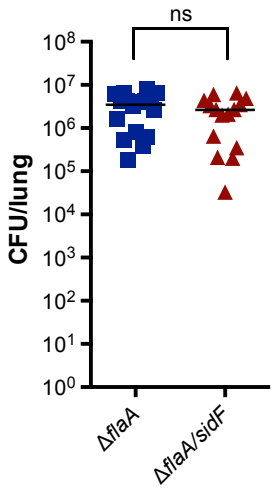


Figure 3.19 SidF is not required for *L. pneumophila* infection *in vivo* Bacterial burdens (CFU/lung) collected from the lungs of C57BL/6 mice 48 h after infection with 2.5×10^6 Δ*flaA* and Δ*flaA*/Δ*sidF L. pneumophila*. Mean shown. Each data point represents one mouse.

3.2.15 Absence of the putative SidF targets Bcl-rambo and BNIP3 does not affect in vitro L. pneumophila infection

SidF has been shown to target the pro-apoptotic BH3-only proteins Bcl-rambo and BNIP3 (Banga *et al.*, 2007), both of which may also induce caspase-independent forms of cell death (Rikka *et al.*, 2011; Kim *et al.*, 2012). To test whether Bcl-rambo or BNIP3 can act to disrupt or enhance *L. pneumophila* infection, live-cell imaging was used to quantify cell death over 72 h in WT C57BL/6, BCL-RAMBO$^{-/-}$, and BNIP3$^{-/-}$ BMDMs infected with the *L. pneumophila* strains Δ*flaA* (Fig. 3.20a) or Δ*flaA*/Δ*sidF* (Fig. 3.20b). All of the uninfected and the Δ*dotA*-infected BMDMs remained viable for 30 h and only showed ~30 % cell death at later time points (Fig. 3.20c and d). In contrast, the death curves for both the Δ*flaA*- and Δ*flaA*/Δ*sidF*-infected WT, BCL-

RAMBO$^{-/-}$, and BNIP3$^{-/-}$ BMDMs were all very similar, with approximately 70 – 80 % of all BMDMs Draq7-positive after 48 h.

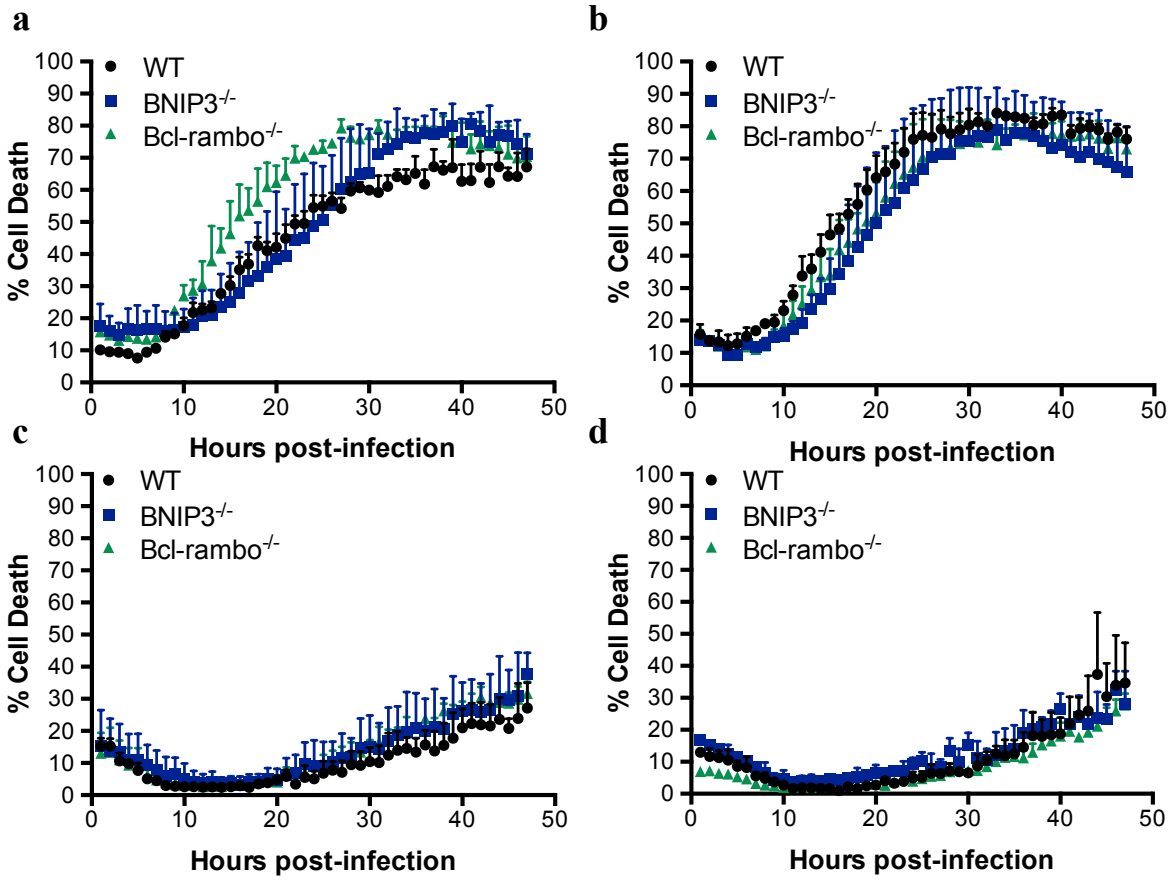


Figure 3.20 Loss of Bcl-rambo and BNIP3 does not affect *in vitro L. pneumophila* infection a, b, c, d) Live-cell microscopic analysis showing percentage cell death over 48 h in Draq7-treated C57BL/6 wild-type (WT), BCL-RAMBO$^{-/-}$, and BNIP3$^{-/-}$ BMDMs infected at a MOI of 10 with the *L. pneumophila* strains **(a)** Δ*flaA*, **(b)** Δ*flaA*/Δ*sidF*, **(c)** Δ*dotA*, or **(d)** in uninfected BMDMs. Data are representative of three independent experiments. Mean and S.D. of three independent biological replicates shown.

3.2.16 Absence of the putative SidF targets Bcl-rambo and BNIP3 does not affect in vivo L. pneumophila infection

Finally, to also confirm that Bcl-rambo and BNIP3 do not contribute to *L. pneumophila* infection *in vivo*, bacterial burdens were calculated from the lungs of WT C57BL/6, BCL-RAMBO$^{-/-}$, and BNIP3$^{-/-}$ mice 48 h after infection with Δ*flaA L. pneumophila* (Fig. 3.21). The results showed that, similar to the *in vitro* data, there was no significant difference in bacterial numbers recovered from the different mouse knock-out strains ($p > 0.05$).

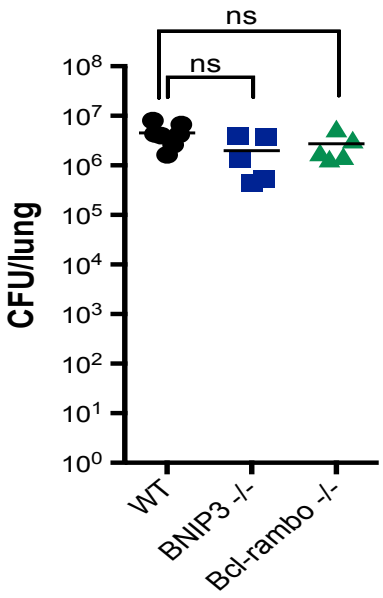


Figure 3.21 Loss of Bcl-rambo and BNIP3 does not affect *in vivo L. pneumophila* infection Bacterial burdens (CFU/lung) collected from the lungs of C57BL/6 wild-type (WT), BNIP3$^{-/-}$, and BCL-RAMBO$^{-/-}$ mice 48 h after infection with 2.5×10^6 Δ*flaA L. pneumophila*. Mean shown. Each data point represents one mouse.

3.3 Discussion

In the work described in this chapter, I established a novel live-cell imaging method to monitor the complex *Legionella*-macrophage interaction, which includes the establishment of a LCV [reviewed in Isberg *et al.*, (2009)], a lag phase of 6 – 8 h, and a replicative phase of an additional 10 – 12 h. By employing a fully-automated microscope with a heated CO_2 chamber to resolve the long-term health of individual infected macrophages, I was able to systematically examine the involvement of different macrophage cell death pathways in the late stages of *Legionella* infection and during bacterial egress. While effective escape from spent host cells contributes to bacterial pathogenesis by promoting disease progression, the mechanism underlying how *Legionella* egress from macrophages has not been investigated in great detail. Here, I have determined the contribution of three host cell death pathways to *Legionella* infection (Tables 3.1 and 3.2).

3.3.1 Pyroptosis

Although flagellin and motility are important for colonisation of protozoan hosts (Pereira *et al.*, 2011), flagellated *Legionella* induce a highly lytic pyroptotic cell death pathway in murine BMDMs, which facilitates efficient escape, but also abrogates replication (Miao *et al.*, 2010). Flagellin, which is thought to translocate into the cytosol via the Dot/Icm T4SS, largely due to its high level of expression (Ren *et al.*, 2006), is sensed by the NAIP5/NLRC4/caspase-1 inflammasome axis. However, there is limited evidence that *Legionella* actually use this pathway to egress from macrophages, as: 1) flagellin-deficient (Δ*flaA*) *L. pneumophila* still causes host cell death and replicates efficiently; 2) *L. longbeachae*, which is naturally aflagellated, still induces cell death and lethal pneumonia; and 3) flagellin-mediated cell death also

induces a rapid anti-bacterial response, contingent on Il-1β secretion and the recruitment of activated neutrophils.

Legionella also activate a caspase-11-dependent form of pyroptotic cell death, particularly in LPS-primed cells, to induce caspase-11 expression (Case *et al.*, 2013). Cytosolic caspase-11 recognises and binds lipopolysaccharide (LPS) directly to either induce NLRP3/caspase-1 dependent or caspase-1 independent pyroptosis (Hagar *et al.*, 2013; Kayagaki *et al.*, 2013). Although caspase-11 is not required for NLRC4-dependent pyroptosis, nor for the restriction of flagellated *Legionella* infection (Cerqueira *et al.*, 2015), there is some evidence that *Legionella* complete their terminal rounds of proliferation within the cytosol (Molmeret *et al.*, 2010) and, thus, may activate caspase-11 upon escaping the LCV, in order to facilitate egress.

The lag in cell death observed at a low MOI in Δ*flaA L. pneumophila*-infected caspase-11-deficient BMDMs, supports the idea that cytosolic *L. pneumophila* may activate caspase-11 to escape from spent cells. Other vacuolar pathogens also induce caspase-11-mediated death to effectively escape. For example, caspase-11 activation in response to *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection is detrimental to the host as it expedites bacterial egress, allowing *S.* Typhimurium to replicate extracellularly in the absence of a caspase-1-mediated immune response (Broz *et al.*, 2012). However, because the delay in cell death in the absence of caspase-11 is seen only at low infection levels, higher numbers of bacteria must be able to trigger escape independently of, and more quickly than, caspase-11 activation alone. Furthermore, even at a low MOI, there is no corresponding defect in bacterial replication or escape, indicating that this delay in cell death does not impair *Legionella* egress sufficiently to limit bacterial replication. Thus, while cytosolic *Legionella* may

activate caspase-11-mediated cell death in the late stages of infection, there is limited evidence that it promotes bacterial egress. In examining bacterial escape from spent macrophages, however, it should be noted that, even under conditions where all host cells are dead, large numbers of *Legionella* remain associated with the cell pellet fraction, rather than the culture media. This could be because macrophages are inherently 'sticky' cells, and bacteria may remain adherent without being 'released' into the supernatant. Thus, alternative assays that are able to quantitatively detect bacterial escape are needed to clarify the role of caspase-11 during *Legionella* egress.

Flagellated WT *L. pneumophila* still induced rapid cell death and failed to replicate efficiently in C57BL/6-derived Caspase-$1^{-/-}$/Caspase-$11^{-/-}$ BMDMs, supporting prior reports that *Legionella* can induce an alternative form of cell death that is dependent on flagellin and NLRC4 activation, but independent of both caspase-1 and caspase-11 (Pereira *et al.*, 2011; Cerqueira *et al.*, 2015). While the exact nature of this cell death pathway has not yet been elucidated, one possible factor is caspase-8, which can be recruited and activated by inflammasomes (Gurung and Kanneganti, 2015). Notably, both the robustness and rapidity of this cell death response, compared with that seen with the Δ*flaA* strain, suggest that it may play a major, albeit hidden, role in *Legionella* infection. This runs counter to previous studies using caspase-1/11-deficient BMDMs, which have focused primarily on the first 4 h of infection and show only minimal cell death (Ren *et al.*, 2006; Cerqueira *et al.*, 2015).

One unexpected observation was the extent to which WT *L. pneumophila* infection also induced rapid cell death in A/J-derived BMDMs, which are reportedly permissive for *L. pneumophila* infection, due to a defective *NAIP5* allele (Diez *et al.*, 2003; Wright Jr *et al.*, 2003; Ren *et al.*, 2006). It is possible that the flagellin/NLRC4-

dependent, caspase-1/11 independent pathway, described above in C57BL/6-derived BMDMs, may also be active in A/J-derived BMDMs, especially as the involvement of NAIP5 in this has not been determined. However, A/J-derived BMDMs can still fully, or almost fully, activate caspase-1 in response to flagellated *L. pneumophila* infection (Zamboni *et al*., 2006; Lamkanfi *et al*., 2007b). In contrast, *L. pneumophila* infection of NAIP5-deficient macrophages does not activate caspase-1-mediated cell death (Lightfield *et al.*, 2008), supporting previous reports that the NAIP5 allele in A/J mice is still partially functional. Thus, this rapid cell death observed following WT *L. pneumophila* infection may be due primarily to NLRC4 and caspase-1 activation.

The extent to which WT *L. pneumophila* activates caspase-1-dependent cell death may depend, in large part, upon the degree of flagellin-mediated NAIP5 activation. This could be linked to the sensitivity of different NAIP proteins, and/or to the amount of flagellin translocated into the cytosol by different *Legionella* serogroups. Here, the rapid cell death observed in the A/J-derived BMDMs infected with WT *L. pneumophila* suggests that the 130b strain, which contains overlapping, but also distinct, sets of effectors from other *L. pneumophila* serogroups (Schroeder *et al.*, 2010), may induce NAIP5 activation more robustly.

Table 3.1 Cell death and *Legionella* growth in macrophages I

Macrophage line		**Rapid Cell Death (Draq7 uptake within 24 h)**			**Growth Restriction**		
Name	Genotype	*L.pn*	*L.pn* Δ*flaA*	*L.bch*	*L.pn*	*L.pn* Δ*flaA*	*L.bch*
C57BL/6	*Caspase-1*$^{+/+}$ *Caspase-11*$^{+/+}$ *NAIP5*^{R}	++	-	-	+	-	-
Caspase-1/11	*Caspase-1*$^{-/-}$ *Caspase-11*$^{-/-}$ *NAIP5*^{R}	++	-	-	+	-	-
Caspase-11	*Caspase-1*$^{+/+}$ *Caspase-11*$^{-/-}$ *NAIP5*^{R}	++	-	ND	ND	ND	ND
A/J	*Caspase-1*$^{+/+}$ *Caspase-11*$^{+/+}$ *NAIP5*^{P}	++	ND	ND	ND	ND	ND

Expected

Unexpected

^{R} = Repressive

^{P} = Permissive

ND: not determined

3.3.2 Necroptosis

Necroptotic cell death is increasingly being recognised as an important mediator of host cell defence against infection, and many pathogens also manipulate this pathway to aid in their dissemination [reviewed in Sridharan and Upton, (2014)]. For example, *Mycobacterium tuberculosis* invades alveolar macrophages in the human lung and induces tumour necrosis factor (TNF) expression. Although excess TNF initially aids

in control of the bacteria by causing a RIPK1/RIPK3-dependent increase in reactive oxygen species (ROS), it eventually results in necroptotic cell death that facilitates release of *M. tuberculosis* into the extracellular space (Roca and Ramakrishnan, 2013). In contrast, *S.* Typhimurium actually uses type I interferon-induced necroptosis of infected macrophages to evade the innate immune system, at least under certain conditions (Robinson *et al.*, 2012). Investigation into the involvement of necroptosis during *Legionella* infection revealed that, in the absence of the essential necroptotic factors RIP3K and MLKL, both *L. pneumophila* and *L. longbeachae* were able to replicate and induce cell death normally, both *in vitro* and in the lungs of experimental mice. Thus, in contrast to other intracellular pathogens, there is little evidence so far that *Legionella* induce necroptotic cell death mediated by RIPK3 and MLKL.

3.3.3 Apoptosis

Because *Legionella*-infected cells have been reported to contain high levels of active caspase-3 and often appear apoptotic in the late stages of infection, despite their resistance to pro-apoptotic stimuli (Gao and Abu Kwaik, 1999b; Abu-Zant *et al.*, 2005), it has been hypothesised that *Legionella* might use caspase-3-mediated apoptosis to facilitate egress during the late stages of infection (Molmeret and Abu Kwaik, 2002). However, the data in this chapter clearly demonstrate that abolition of canonical apoptosis, either via chemical inhibition of the executioner caspases or via loss of the essential mitochondrial apoptotic effectors, BAX and BAK, does not conspicuously affect the ability of *Legionella* to induce cell death and egress normally. This suggests that, while *Legionella* may modify anti-apoptotic signalling during the course of infection in order to ensure their survival, they do not require mitochondrial apoptosis to initiate egress.

During the early stages of *Legionella* infection in human macrophages, an alternative role for active caspase-3 in evading the endosomal-lysosomal degradation pathway has also been reported (Molmeret *et al.*, 2004b). However, pre-treatment of BMDMs with the pan-caspase inhibitor Q-VD did not result in diminished *Legionella* replication. This result supports the report that *Legionella* replicate normally in caspase-3-deficient BMDMs (Nogueira *et al*., 2009) and, together, suggest that this putative role for caspase-3 in *Legionella* infection does not carry across into murine macrophages.

Although the data suggest that caspase-3 is not critically important to *Legionella* infection in murine macrophages, it is possible that it plays an essential role during the infection of alternative host cells, *e.g.,* dendritic cells (DCs). DCs are more sensitive than macrophages to cell death induced by *Legionella* (Nogueira *et al.*, 2009), and a *Legionella* mutant lacking five effectors that partially induce caspase-3 activation is less able to trigger apoptotic cell death in DCs, although there is no corresponding defect in macrophages (Zhu *et al.*, 2013). This suggests that *Legionella* may actively induce apoptosis in DCs in order to prevent activation of adaptive immunity and/or dissemination to more hostile organs.

A previous study had reported a small but significant growth defect in BMDMs with *L. pneumophila* strains lacking SidF (Banga *et al.*, 2007), and the data in this chapter support this with approximately 3-fold fewer Δ*flaA*/Δ*sidF* bacteria recovered from BMDMs at 48 h post-infection, compared with the Δ*flaA* strain. However, no corresponding replication defect was observed *in vivo* with the Δ*flaA*/Δ*sidF* strain compared to the Δ*flaA* strain. Furthermore, contrary to the data published by Banga *et al.* (2007), no increase in apoptotic cell death was observed during any stage of macrophage infection with an *L. pneumophila* strain lacking SidF. There was no

detectable difference at all in the rate or extent of cell death seen with the Δ*flaA*/Δ*sidF* strain, compared to the Δ*flaA* strain. Finally, no difference was observed between Δ*flaA L. pneumophila* infection in BMDMs, or in mice, lacking either of the putative SidF target BH3-only proteins, Bcl-rambo or BNIP3, compared with WT BMDMs and mice. It is possible that this discrepancy is caused by that fact that Banga *et al*. (2007) used a different *L. pneumophila* strain, Philadelphia 1 (Lp02) that infects and replicates more robustly than the 130b strain, which might exacerbate the cell death phenotype.

Altogether, these data suggest that: 1) SidF does not play a critical role in preventing apoptotic cell death promoted by Bcl-rambo and BNIP3 during *L. pneumophila* infection; and 2) if Bcl-rambo and BNIP3 are involved in pro-apoptotic signalling during *L. pneumophila* infection, then their roles are wholly redundant. To show that SidF interacts with Bcl-rambo and BNIP3, Banga *et al.* (2007) used yeast-two-hybrid and co-immunoprecipitation assays, both of which are techniques prone to generating false-positive results. More recently, it has been shown that SidF is a phosphatidylinositol polyphosphate 3-phosphatase that specifically hydrolyses the D3 phosphate of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Hsu *et al.*, 2012). This activity is necessary for anchoring of PI(4)P-binding effectors to bacterial phagosomes. Thus, it is possible that SidF does not interact directly with Bcl-rambo and BNIP3 to mediate apoptotic signalling but, rather, is involved in establishing the lipid profile of the LCV. The prospect that SidF is not a critical modulator of host apoptotic cell death is further supported by the fact that SidF has no apparent homologue in *L. longbeachae* (Gobin *et al.*, 2009).

Table 3.2 Cell death and *Legionella* growth in macrophages II

Macrophage Line		**Cell Death Similar to Wild-type BMDMs**		**Growth Restriction**	
Name	Genotype	*L.pn* Δ*flaA*	*L.bch*	*L.pn* Δ*flaA*	*L.bch*
RIP3	*RIP3*$^{-/-}$ *Caspase-8*$^{+/+}$ *MLKL*$^{+/+}$	+	+	-	-
RIP3/Caspase-8	*RIP3*$^{-/-}$ *Caspase-8*$^{-/-}$ *MLKL*$^{+/+}$	+	+	-	-
MLKL	*RIP3*$^{+/+}$ *Caspase-8*$^{+/+}$ *MLKL*$^{-/-}$	+	+	-	-
BAX/BAK	*BAX*$^{-/-}$ *BAK*$^{-/-}$	+	ND	-	ND
BAK	*BAX*$^{+/+}$ *BAK*$^{-/-}$	+	ND	-	ND
Cyclophilin-D	*Ppif*$^{-/-}$	+	ND	ND	ND
BNIP3	*BNIP3*$^{-/-}$	+	ND	ND	ND
Bcl-rambo	*BCL-RAMBO*$^{-/-}$	+	ND	ND	ND

ND: Not determined

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Writing/Editing	75 %
Experimental Work	95 %

The following co-authors contributed to the work:

Name	**Nature of contribution**
Thomas Naderer	Writing/Editing
James Vince	Writing/Editing
Kate Lawlor	Experimental Work

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature	[redacted]	**Date** 16/11/2015

CHAPTER 4

GENETIC OR PHARMACOLOGICAL TARGETING OF BCL-XL CONTROLS *LEGIONELLA* BY INDUCING APOPTOSIS OF INFECTED MACROPHAGES

Chapter presented as a manuscript submitted to the *Journal of Experimental Medicine*

The previous chapter focused on macrophage cell death pathways and their role in the late stages of infection. From these studies, it has become clear that *Legionella* must evade rapid host cell death to ensure sufficient time for replication. Thus, the present chapter will focus on the effect of inducing apoptotic cell death during the early and replicative phases of intracellular *Legionella* infection, and the subsequent consequences for both host and pathogen.

Genetic or pharmacological targeting of BCL-XL controls *Legionella* by inducing apoptosis of infected macrophages

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Abstract

Programmed death of host cells can deprive obligate intracellular bacterial pathogens of their replicative niche and expose them to immune attack (Nogueira *et al.*, 2009; Miao *et al.*, 2010; Martin *et al.*, 2012; Aachoui *et al.*, 2013). The intracellular pathogen *Legionella* causes lethal pneumonia (Newton *et al.*, 2010) by replicating in alveolar macrophages, and may actively suppress host cell apoptosis to facilitate bacterial growth (Losick and Isberg, 2006; Abu-Zant *et al.*, 2007; Banga *et al.*, 2007). Here, we demonstrate that expression of the pro-survival BCL-2 family member, BCL-XL, is critical for *Legionella* to prevent apoptosis in infected macrophages. In the absence of BCL-XL, macrophages that become infected with *Legionella* undergo rapid apoptotic cell death, which abolishes intracellular bacterial replication and dissemination. Similarly, BH3-mimetic compounds that target BCL-XL, but not BCL-2, induce apoptosis of *Legionella*-infected macrophages to limit bacterial growth, whereas uninfected macrophages remain viable. Impaired survival of infected macrophages and reduced *Legionella* burdens due to loss of BCL-XL can be restored by inhibiting mitochondrial apoptosis. Remarkably, *Bcl-x* gene deletion, or a single dose of BCL-XL-targeted BH3-mimetic therapy, significantly reduces *Legionella* burden in the lungs and prevents lethal bacterial infection in mice. These results demonstrate that *Legionella*-infected macrophages are specifically and exquisitely sensitive to apoptotic cell death following the loss or inhibition of BCL-XL. Consequently, repurposing of anti-cancer drugs, such as BH3-mimetic compounds, to target specific host pro-survival factors may represent a promising strategy for the treatment of infections involving intracellular pathogens.

BCL-2 is the prototypical pro-survival member of the BCL-2 protein family, which also includes BCL-XL, BCL-w, MCL-1, and A1 (Strasser *et al.*, 2011). These pro-survival proteins bind directly to BAK and BAX to prevent BAK/BAX oligomerisation, pore formation on the outer mitochondrial membrane, and release of cytochrome-*c* (Ola *et al.*, 2011). Cytosolic cytochrome-*c* triggers activation of the initiator caspase, caspase-9, which, in turn, activates executioner caspases, such as caspase-3, resulting in apoptotic cell death. Pro-survival BCL-2 family members are potently and specifically antagonised by pro-apoptotic BH3-only proteins and, consequently, BH3-mimetic compounds have been developed to promote cancer-cell apoptosis (van Delft *et al.*, 2006). However, their potential therapeutic use in other settings, particularly in microbial infectious diseases, remains unexplored.

The intracellular pathogen *Legionella pneumophila* establishes a replicative niche in permissive macrophages, which develop resistance to apoptosis-inducing stimuli, such as staurosporine and TNF-α (Abu-Zant *et al.*, 2007). Among the large set of *Legionella* effector molecules exported by the type IV secretion system (T4SS) (Zhu *et al.*, 2011; Dolezal *et al.*, 2012; Lifshitz *et al.*, 2013), SidF has been reported to inhibit the putative pro-apoptotic proteins BNIP3 and Bcl-rambo (Banga *et al.*, 2007). Other effectors are thought to induce the transcriptional up-regulation of pro-survival factors, including BCL-2 and A1 (Losick and Isberg, 2006; Abu-Zant *et al.*, 2007). In bone marrow-derived macrophages (BMDMs) from C57BL/6 mice, however, wild-type *L. pneumophila* triggers flagellin-mediated, caspase-1-dependent cell death (termed pyroptosis) (Miao *et al.*, 2010; Zhao *et al.*, 2011), which prevents robust intracellular bacterial replication (Amer *et al.*, 2006; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Zamboni *et al.*, 2006).

To investigate the role of BCL-2 in *L. pneumophila* infection, we used the flagellin-deficient Δ*flaA* mutant strain, as Δ*flaA*-infected BMDMs remain viable for up to 20 h post-infection to allow for intracellular replication (Figure 1a). Subsequent macrophage death occurred in ~80 % of BMDMs by 60 h post-infection (Figure 1a), reflecting continual cycles of bacterial replication, release, and re-infection.

The BH3-mimetic compound, ABT-737, antagonises pro-survival BCL-2 family members to cause cell death in some cells (Lessene *et al.*, 2008). Unexpectedly, when Δ*flaA L. pneumophila*-infected BMDMs were treated with ABT-737, the majority of macrophages (~65 %) were protected from the extensive cell death observed in untreated Δ*flaA*-infected cells by 60 h post infection (Figure 1a). Similarly, ABT-737-treatment also protected BMDMs from extensive cell death after infection with wild-type *L. pneumophila* (Extended Data Figure 1b). In both cases, ABT-737 treatment resulted in the death of 30 – 35 % of BMDMs (Figure 1a and Extended Data Figure 1b), in the early stage of infection. Notably, uninfected BMDMs remained viable (Extended Data Figure 1a), and ABT-737-treatment also did not induce significant death in BMDMs infected with the avirulent Δ*dotA L. pneumophila* strain lacking a functional T4SS (Figure 1b), nor did it alter *Legionella* axenic growth (data not shown).

We hypothesised that ABT-737 might induce selective killing of the initial *Legionella*-harbouring macrophages (~30 – 40 % of cells) shortly after bacterial invasion. Consistent with this idea, at higher infection rates ABT-737-treatment resulted in the rapid killing of ~80 % of BMDMs in culture, which occurred faster than Δ*flaA L. pneumophila*-mediated death in macrophages that had not been exposed to ABT-737 (Figure 1c). Moreover, live-cell imaging of immortalised C57BL/6 (iBl/6)

macrophages after infection with GFP-expressing Δ*flaA L. pneumophila* demonstrated that ABT-737-treatment rapidly induced apoptotic morphology (plasma membrane ruffling and blebbing) followed by plasma membrane rupture (determined by Draq7 staining) only in GFP positive macrophages (Figure 1d and supplementary Movies 1 and 2). In the absence of ABT-737, this apoptotic morphology was not observed and Δ*flaA L. pneumophila* did not induce significant macrophage death within 12 h, despite robust intracellular bacterial replication, visualised by increasing GFP fluorescence (Figure 1d and supplementary Movie 2). In contrast, in the presence of ABT-737, GFP fluorescence (indicative of bacterial replication) failed to increase over the 48 h following infection (Figure 1d and supplementary Movie 1).

To examine directly whether ABT-737-treatment reduces intracellular *L. pneumophila* loads, we measured colony-forming units (CFUs). The Δ*fla*A *L. pneumophila* strain assumes exponential growth approximately 6 h post-infection (Figure 1e). While ABT-737-treatment did not affect the initial macrophage infection rate within this time-frame (Figure 1e), it prevented any subsequent (24 and 48 h post-infection) increase in intracellular Δ*flaA L. pneumophila* burdens (Figure 1e). Collectively, these data demonstrate that ABT-737-treatment specifically induces the death of macrophages containing virulent *Legionella*, and that this limits the intracellular bacterial burden.

ABT-737 targets BCL-2, BCL-W, and BCL-XL (Figure 2a). To gain insight into which BCL-2 member protects *Legionella*-infected macrophages from death, we utilised BH3-mimetic compounds that specifically bind to BCL-2 or BCL-XL. Surprisingly, the BCL-2-specific antagonist, ABT-199, failed to clear GFP-expressing Δ*flaA L. pneumophila* in iBL/6 and BMDM host cells (Figure 2a, b and c). In contrast to ABT-199, the BCL-XL-specific inhibitor A-1155463, which does not antagonise

BCL-2 or BCL-W significantly (Tao *et al.*, 2014), mimicked the effect of ABT-737. Specifically, similar to ABT-737, A-1155463-treatment resulted in a loss of GFP signal in iBl/6 macrophages after infection with GFP-expressing Δ*flaA L. pneumophila*, and resulted in a > 100-fold CFU reduction in BMDMs, at 48 h post infection (Figure 2a and c). Consequently, A-1155463 protected macrophages from extensive *Legionella*-mediated killing (Figure 2a and b). Remarkably, A-1155463 was effective in limiting Δ*flaA L. pneumophila* burdens even at low nanomolar concentrations, whereas ABT-199 was inefficient at doses lower than 5 μM (Figure 2d). The efficacy of these BH3-mimetics at restricting *L. pneumophila* infection in human THP-derived macrophages was also tested (Extended Fig. 2a). Surprisingly, while neither ABT-199 nor A-1155463-treatment significantly limited *L. pneumophila* replication, ABT-737 was able to block replication, suggesting that BCL-2 and BCL-XL may have redundant roles during *L. pneumophila* infection of human cells.

To genetically confirm a role for BCL-XL in promoting *Legionella* infection of murine macrophages, we generated BCL-XL-deficient BMDMs. Constitutive loss of BCL-XL causes embryonic lethality around E14 due to excessive apoptosis of erythroid and neuronal progenitors (Motoyama *et al.*, 1995). Thus, we treated bone marrow progenitor cells from *Bcl-x*$^{flox/flox;}$*;ER-Cre* mice, which contain two floxed *Bcl-x* alleles (Wagner *et al.*, 2000) and express the Cre-recombinase oestrogen receptor fusion protein (Rosa26-Cre-ERT2), with 4-hydroxytamoxifen (4-HT) to induce deletion of BCL-XL (referred to as *Bcl-x*$^{-/-}$). This resulted in a > 90 % reduction of BCL-XL-protein expression in differentiated BMDMs (Extended Data Figure 2c). Importantly, genetic deletion of *Bcl-x* mimicked BCL-XL-antagonism by BH3-mimetic compounds. First, the initial infection of *Bcl-x*$^{-/-}$ BMDMs with Δ*flaA L. pneumophila*

was not altered when compared to wild-type macrophages, but subsequent *Legionella* burdens decreased over time (Figure 2e). Second, *Bcl-x*-gene deletion protected the bulk of the macrophage population (~80 %; *i.e.*, those not initially infected) from Δ*flaA L. pneumophila*-induced cell lysis when macrophages were infected at a low MOI (Figure 2f). Third, *Bcl-x*$^{-/-}$ BMDMs were resistant to wild-type *L. pneumophila*-induced cell death (Extended Data Figure 2d). As expected, *Bcl-x*$^{-/-}$ BMDMs remained viable after infection with the avirulent Δ*dotA L. pneumophila* strain (Figure 2g). Collectively, these data demonstrate that BCL-XL is uniquely required in *Legionella*-infected macrophages to sustain host cell survival and allow for efficient intracellular bacterial replication.

One of the functions of BCL-XL is to inhibit the mitochondrial pathway of apoptosis that culminates in executioner caspase-3 activation (Strasser *et al.*, 2011). Consistent with this, Δ*flaA,* but not Δ*dotA, L. pneumophila*-infected *Bcl-x*$^{-/-}$ BMDMs showed increased caspase-3 activation compared to wild-type BMDMs, as measured by immunoblotting or fluorescence-staining of the active caspase-3 p17/p19 fragment (Figure 3a and c). These data suggest that caspase-mediated apoptosis following BH3-mimetic treatment prevents intracellular *Legionella* growth.

Therefore, to test if *Legionella* growth could be restored in the absence of BCL-XL activity by inhibiting caspase-mediated apoptosis, we treated Δ*flaA L. pneumophila*-infected macrophages deficient in *Bcl-x* with the pan-caspase inhibitor Q-VD-OPh (Q-VD). By itself, Q-VD had no effect on the intracellular burden of Δ*flaA L. pneumophila* (Figure 3c) but, remarkably, it restored bacterial numbers in *Bcl-x*$^{-/-}$ BMDMs (Figure 3h). Similarly, Q-VD also restored bacterial numbers in ABT-737- and A-1155463-treated wild-type BMDMs (Figure 3c). Given that Q-VD restored

bacterial replication in the presence of ABT-737, it should also restore the efficient killing of macrophages by Δ*flaA*. Replicating Δ*flaA L. pneumophila* eventually initiate macrophage lysis that is not blocked by Q-VD, in order to egress and, therefore, this is likely to occur independently of caspase function (Figure 3d). As expected, Q-VD efficiently restored the ability of Δ*flaA L. pneumophila* to lyse BMDMs in the presence of either of the BCL-XL-targeting BH3-mimetics, ABT-737 or A-1155463 (Figure 3f and g). Q-VD had little effect on bacterial load or host cell death in Δ*flaA L. pneumophila*- infected wild-type BMDMs treated with ABT-199 (Figure 3c and e).

To prove genetically that mitochondrial apoptosis is responsible for limiting *L. pneumophila* growth in the absence of BCL-XL, we examined macrophages deficient in the essential mitochondrial apoptotic executioners, BAX and BAK. Combined BAX/BAK-deficiency did not affect intracellular Δ*flaA L. pneumophila* load (Figure 3i). Notably however, BAX/BAK loss restored Δ*flaA L. pneumophila* load in ABT-737- and A-1115463-treated BMDMs to levels that were seen in infected wild-type (or $BAX^{-/-}/BAK^{-/-}$) BMDMs that had been treated with vehicle only (Figure 3i). In contrast, Δ*flaA L. pneumophila*-induced killing of macrophages was not abrogated in BMDMs deficient in the pyroptotic caspases-1 and -11 (Extended Data Figure 3 b), the necroptotic factors, RIPK3 and MLKL, nor the extrinsic apoptotic factor, caspase-8 (Extended Data Fig 3c, d, e, f). Similar to WT BMDMs (Extended Fig. 3a), Δ*flaA L. pneumophila* was less able to induce cell death in the ABT-737-treated BMDMs than in the untreated BMDMs. Collectively, these data demonstrate that BCL-XL is essential for intracellular *Legionella* infection and replication because it prevents BAX/BAK-dependent mitochondrial apoptosis of infected cells.

To test whether selective targeting of BCL-XL reduces bacterial load in the lungs *in vivo*, we treated > 10-week-old *Bcl-x$^{flox/flox}$;ER-Cre* mice with tamoxifen to generate BLC-XL-deficient mice. These mice contained normal numbers of bone marrow progenitor cells that generated BMDMs lacking detectable levels of BCL-XL protein (Figure 4a) and, as expected, showed reduced bacterial loads 48 h after infection with Δ*flaA L. pneumophila* (Extended Data Figure 3d). Next, tamoxifen-treated or untreated *Bcl-x$^{flox/flox}$;ER-Cre* mice were intranasally infected with Δ*flaA L. pneumophila,* and the bacterial burden in the lungs determined at 48 h post-infection. As expected, after 48 h, untreated *Bcl-x$^{flox/flox}$;ER-Cre* mice showed similar numbers of Δ*flaA L. pneumophila* as C57BL/6 (wild-type control) mice (Figure 4b). In contrast, the bacterial burden was significantly ($p < 0.01$) reduced in tamoxifen-treated *Bcl-x$^{flox/flox}$;ER-Cre* infected mice (Figure 4b). Strikingly, four of 11 mice failed to produce culturable Δ*flaA L. pneumophila*, even after extended incubation. Tamoxifen treatment also did not affect Δ*flaA L. pneumophila* load in the absence of ER-Cre (Figure 4b).

To examine whether pharmacological targeting of BCL-XL reduces *Legionella* lung burden, we infected A/J mice, which are susceptible to wild-type *L. pneumophila.* Immediately after intranasal infection, mice were treated with a single clinically relevant (50 mg/kg body weight) dose of the orally available analogue of ABT-737 (ABT-263). This resulted in a significant reduction ($p < 0.01$) in the numbers of bacteria in the lungs at 48 h post-infection (Figure 4c). Given that wild-type *L. pneumophila* causes a self-limiting infection even in susceptible A/J mice (Molofsky *et al.*, 2006), we also tested whether targeting BCL-XL would affect *Legionella longbeachae,* which grows unrestrained in mouse lungs (Asare *et al.*, 2007; Gobin *et al.*, 2009). *L. longbeachae* causes lethal pneumonia by employing a different set of T4SS effectors than *L. pneumophila* (Cazalet *et al.*, 2010). Despite this, ABT-737 and

A-1155463 both prevented increased intracellular loads of *L. longbeachae* in BMDMs (Extended Data Figure 4a). Administration of ABT-263 effectively controlled the burden of *L. longbeachae* in the lungs of C57BL/6 mice, and we failed to culture any CFUs in four out of ten mice after 72 h of infection (Figure 4d). Furthermore, a single 50 $mg.kg^{-1}$ dose of ABT-263 completely rescued C57BL/6 mice from lethal *L. longbeachae* infection, whereas all mice treated with vehicle rapidly lost body weight and succumbed within 96 h of infection (Figure 4e, f). Although it is known that ABT-263 depletes platelets (Vogler *et al.*, 2011), a single dose is unlikely to affect platelet counts greatly. Finally, to show that BCL-XL was the correct target, we also tested the efficacy of the BCL-2-specific inhibitor ABT-199 (Fig. 4g), as well as the BCL-XL-specific inhibitor ABT-852 (Fig. 4h). After 72 h, there was no apparent difference between bacterial burdens in the lungs of mice treated with ABT-199 and those left untreated, In contrast, there was a significant difference ($p < 0.01$) between bacterial burdens in the lungs of the mice treated with ABT-852 and those left untreated. Further experiments will be needed to determine whether BH3 mimetics can be used to clear established acute or chronic infections. However, an alternative animal model may be required, as mice succumb to infection too rapidly to be useful in this setting.

Lastly, to shown that BCL-XL was specifically required within alveolar macrophages, we generated a myeloid-specific BCL-XL-deficient mouse line by crossing *Bcl-x*$^{flox/flox}$ mice with *LysM*$^{Cre/Cre}$ mice (Clausen *et al.*, 1999). These mice also contained normal numbers of bone marrow progenitor cells that generated BMDMs lacking detectable levels of BCL-XL protein, which, nonetheless, remained readily detectable in whole lung extracts (Figure 4i). In addition, the deletion of BCL-XL in myeloid cells did not affect other immune cell populations, including lymphocytes, neutrophils, monocytes, eosinophils, and basophils, as well as platelets and red blood cells (Extended Fig. 4b,

c, d). *Bcl-x*$^{flox/flox}$*;LysM-Cre* mice were then intranasally infected with *L. longbeachae* and lung bacterial burdens calculated after 72 h (Fig. 4j). As, expected, the bacterial burden was significantly ($p < 0.01$) reduced in the *Bcl-x*$^{flox/flox}$*;LysM-Cre* mice, compared with the Cre-negative controls, while the heterozygotes showed an intermediate phenotype. Again, we failed to culture any CFUs in four out of the eight mice, even after extended incubation (Fig. 4j). The fact that the pro-inflammatory cytokines, IL-1β and TNF, were at significantly ($p < 0.01$) lower levels in the lungs of the *Bcl-x*$^{flox/flox}$*;LysM-Cre* mice compared to the Cre-negative controls (Fig. 4k), suggests that this bacterial clearance is not mediated by neutrophil recruitment, but rather via apoptosis of infected macrophages. Collectively, these data extend our *in vitro* observations by demonstrating that *Legionella* specifically require the pro-survival activity of BCL-XL in host macrophages, but not in other cells, in order to sustain infection in the lungs of experimental mice.

Conjointly, our results demonstrate that apoptosis-inducing agents, originally designed to induce cancer-cell death and currently in clinical trials for their efficacy as anti-cancer treatments (Lessene *et al.*, 2008), can be potent antimicrobial compounds. As the threat to human health posed by drug-resistant bacteria looms large (Boucher *et al.*, 2009), this discovery promises a novel therapeutic strategy for the control of intracellular pathogens.

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Author contributions

MS designed and essentially performed all experiments and wrote the manuscript. SG and AS designed experiments, generated mice, and contributed to writing the manuscript. LAOR and KM generated mice. KS wrote ImageJ and MetaMorph scripts. RS generated GFP-expressing and mutant *Legionella*. ELH and LT designed experiments and contributed to writing the manuscript. DCSH advised on BH3-mimetics and designed experiments. GL generated the BCL-XL-specific BH3-mimetic. JEV and TN conceived the project, designed and performed experiments, and wrote the manuscript.

Author information

The authors declare that SPG, LAOR, AS, GL, DSCH and JEV are employees of The Walter and Eliza Hall Medical Institute, which receives milestone payments from Genentech and AbbVie for the development of ABT-199 for cancer therapy.

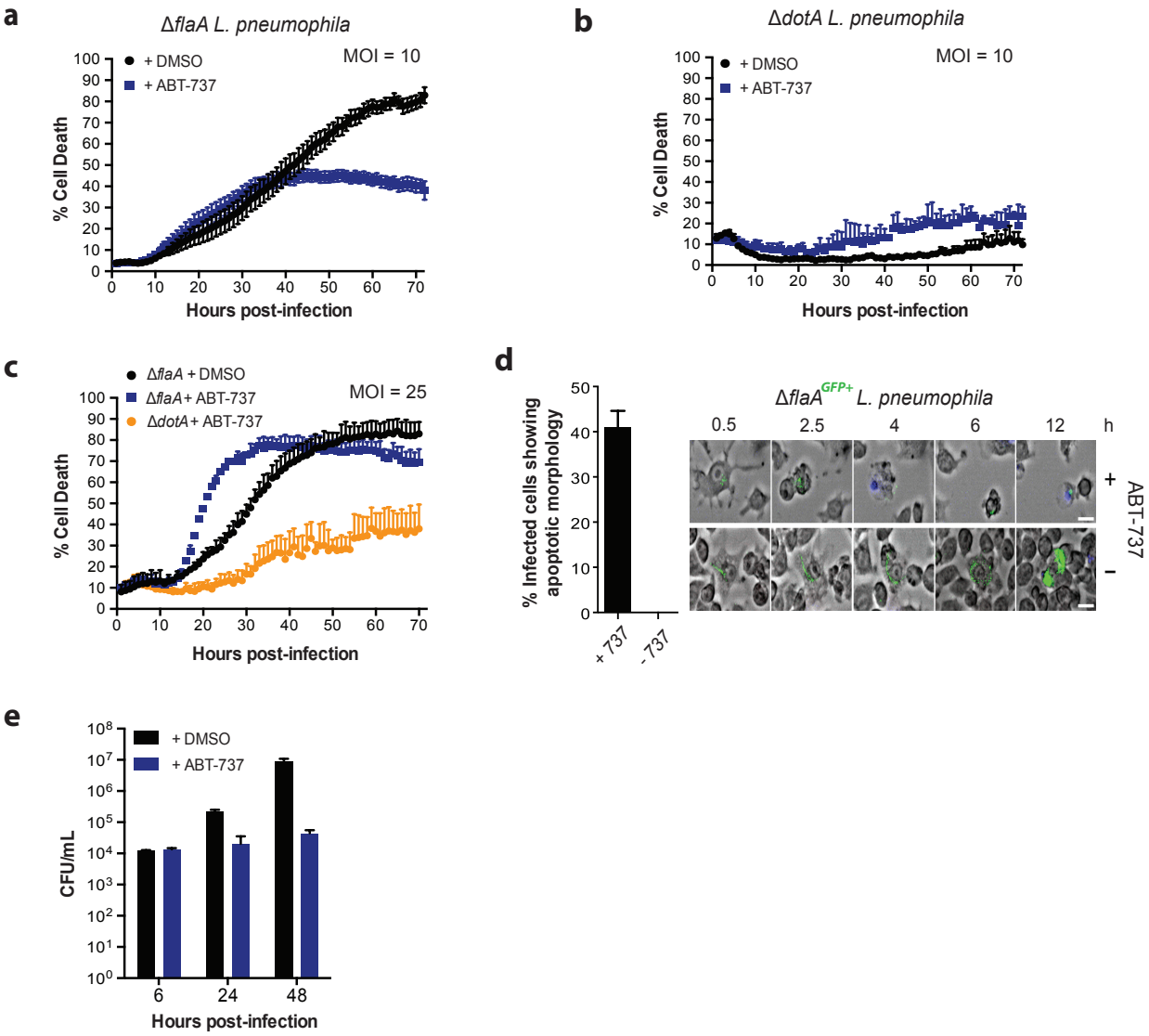


Figure 1. ABT-737 restricts *Legionella* burdens in BMDMs, *in vitro*, by inducing host cell death. a, b, c, Draq7-positive (dead) bone marrow-derived macrophages (BMDMs) were quantified by live-cell imaging over 72 h, following infection with (**a, c**) Δ*flaA* or (**b**) Δ*dotA Legionella* at (**a**, **b**) MOI of 10 or (**c**) 25, and addition of 10 μM ABT-737. Mean and standard deviation (SD) of three repeats are shown. Data representative of at least three independent experiments. **d**, Time-lapse images of immortalized C57BL/6 macrophages infected with Δ*flaA*-expressing GFP, in the presence of Draq7 and ABT-737 (500 nM) or vehicle. Scale bar = 10 μm. Infected macrophages showing apoptotic morphology were quantified 24 h post-infection in three independent samples (a total of 600 cells from triplicate experiments was analysed). **e,** Δ*flaA* replication in BMDMs treated with 500 nM ABT-737 was examined by determining bacterial colony-forming units (CFUs) at the indicated time points. Mean and standard error (SE) are shown (n = 3).

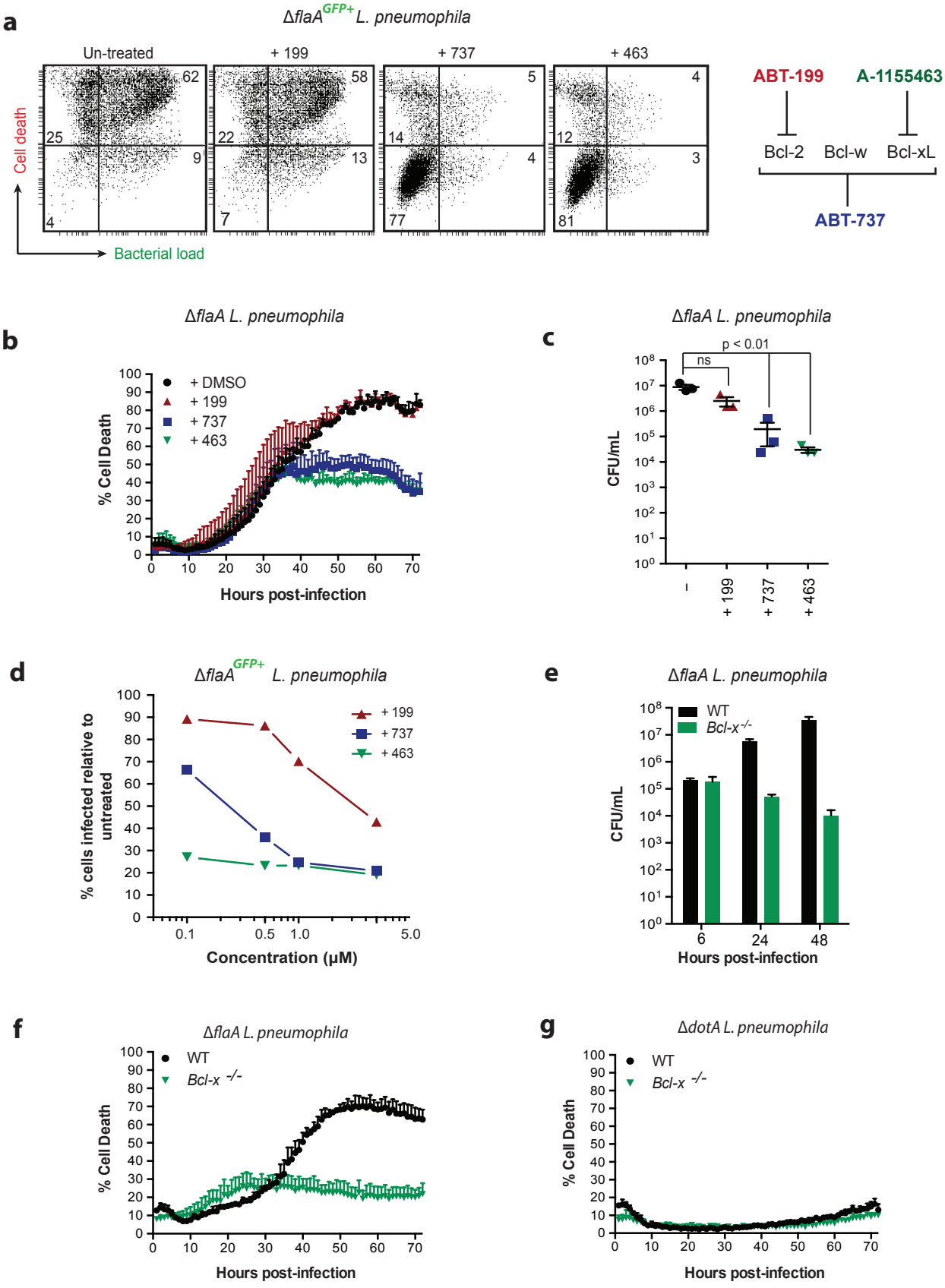
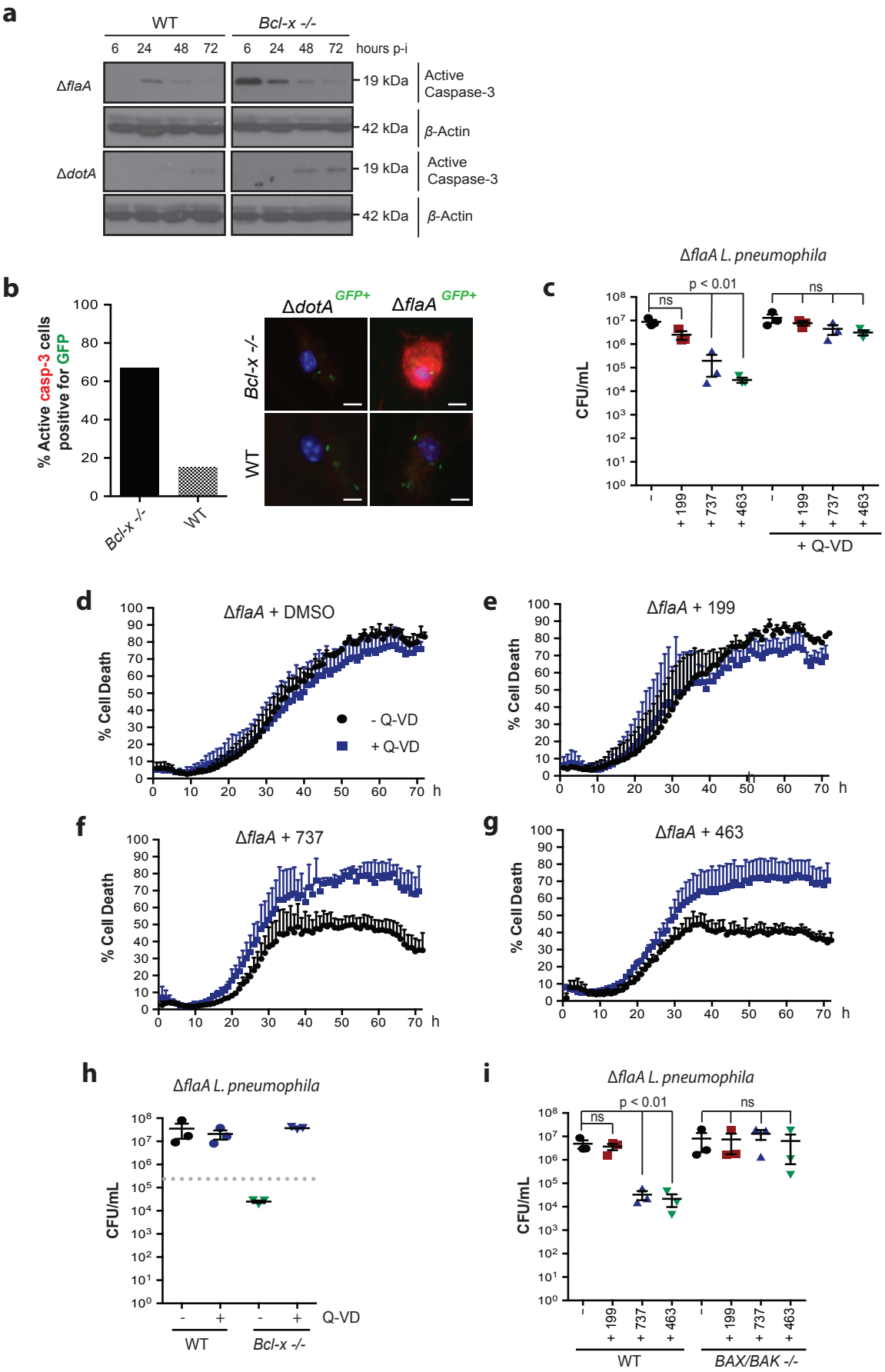

a
ΔflaA GFP+ L. pneumophila
Un-treated
+ 199
+ 737
+ 463
Cell death
Bacterial load
ABT-199
A-1155463
Bcl-2
Bcl-w
Bcl-xL
ABT-737
b
ΔflaA L. pneumophila
+ DMSO
+ 199
+ 737
+ 463
% Cell Death
Hours post-infection
c
ΔflaA L. pneumophila
p < 0.01
ns
CFU/mL
d
ΔflaA GFP+ L. pneumophila
% cells infected relative to untreated
Concentration (μM)
e
ΔflaA L. pneumophila
WT
Bcl-x -/-
CFU/mL
Hours post-infection
f
ΔflaA L. pneumophila
WT
Bcl-x -/-
% Cell Death
Hours post-infection
g
ΔdotA L. pneumophila
WT
Bcl-x -/-
% Cell Death
Hours post-infection

Figure 2. Targeting BCL-XL, rather than BCL-2, limits intracellular *Legionella* loads. a, Left: flow cytometry analysis of cell death (propidium iodide staining) and bacterial load (GFP fluorescence) of Δ*flaA*$^{GFP+}$-infected immortalized C57BL/6 macrophages treated with 500 nM BH3-mimetics, 48 h post-infection (MOI = 10). The percentage of cells in each quartile of a dot plot are shown. Right: schematic depicting the pro-survival BCL-2 family members targeted by the different BH3-mimetics used in this study (A-1155463 is subsequently abbreviated to 463). **b**, Live-cell microscopic analysis of Draq7-positive (dead) bone marrow-derived macrophages (BMDMs) measured for 72 h following infection with Δ*flaA Legionella* and treatment with BH3-mimetics (500 nM). Mean and SD of three repeats are shown. Data representative of at least three independent experiments. **c,** Bacterial burden (CFUs) of Δ*flaA Legionella* isolated from BMDMs treated with BH3-mimetics (500 nM) at 48 h post-infection are shown. Mean (line) and SE are shown (n = 3). **d,** Flow cytometric analysis of GFP+ macrophages infected with Δ*flaA*$^{GFP+}$ and treated with different BH3-mimetic compounds at the indicated concentrations for 48 h. Mean of triplicate samples is shown. **e,** Bacterial burden (CFUs) of Δ*flaA Legionella* isolated from wild-type (WT) and BCL-XL-deficient BMDMs. Mean and SE are shown (n = 3). **f, g,** Live-cell microscopic analysis of Draq7-positive (dead) BMDMs infected with Δ*flaA* and Δ*dotA*. Mean and SD of three repeats are shown. Data representative of at least three independent experiments.

Figure 3. Loss of BCL-XL induces apoptosis in *Legionella*-infected macrophages. a, Time-course immuno-blot analysis for cleaved (*i.e.*, activated) caspase-3 in wild-type (WT) and BCL-XL-deficient bone marrow-derived macrophages (BMDMs) infected with Δ*flaA* or Δ*dotA*. Actin blot is a loading control. Data representative of two independent experiments. **b,** Indirect immuno-fluorescence staining analysis of activated caspase-3 in BMDMs infected with *Legionella* expressing GFP. Scale bar = 10 μm. >200 cells were analysed. **c**, Δ*flaA Legionella* burden (CFUs) of infected BMDMs at 48 h post-infection following treatment with 500 nM of BH3-mimetic and 20 μM of the pan-caspase inhibitor Q-VD-OPh (Q-VD). Mean (line) and SE are shown (n = 3). **d, e, f, g,** Live-cell microscopic analysis of Draq7-positive (dead) BMDMs infected with Δ*flaA* and treated with 500 nM of BH3-mimetic and 20 μM Q-VD-OPh (Q-VD). Mean and SD of three repeats are shown. Data representative of at least three independent experiments. **h, i,** Bacterial burden (CFUs) of Δ*flaA*-infected WT, *Bcl-x*$^{-/-}$, or BAX$^{-/-}$ /BAK$^{-/-}$ BMDMs at 48 h post-infection. Mean (line) and SE are shown (n = 3). Dotted line indicates mean CFUs of untreated BMDMs at 6 h post-infection.


a
WT
Bcl-x -/-
6 24 48 72 6 24 48 72 hours p-i
ΔflaA
19 kDa
Active Caspase-3
42 kDa
β-Actin
ΔdotA
19 kDa
Active Caspase-3
42 kDa
β-Actin
b
% Active casp-3 cells positive for GFP
Bcl-x -/-
WT
ΔdotA GFP+
ΔflaA GFP+
c
ΔflaA L. pneumophila
CFU/mL
p < 0.01
ns
ns
- + 199 + 737 + 463 - + 199 + 737 + 463
+ Q-VD
d
ΔflaA + DMSO
% Cell Death
- Q-VD
+ Q-VD
h
e
ΔflaA + 199
f
ΔflaA + 737
g
ΔflaA + 463
h
ΔflaA L. pneumophila
CFU/mL
- + - + Q-VD
WT
Bcl-x -/-
i
ΔflaA L. pneumophila
p < 0.01
ns
ns
WT
BAX/BAK -/-

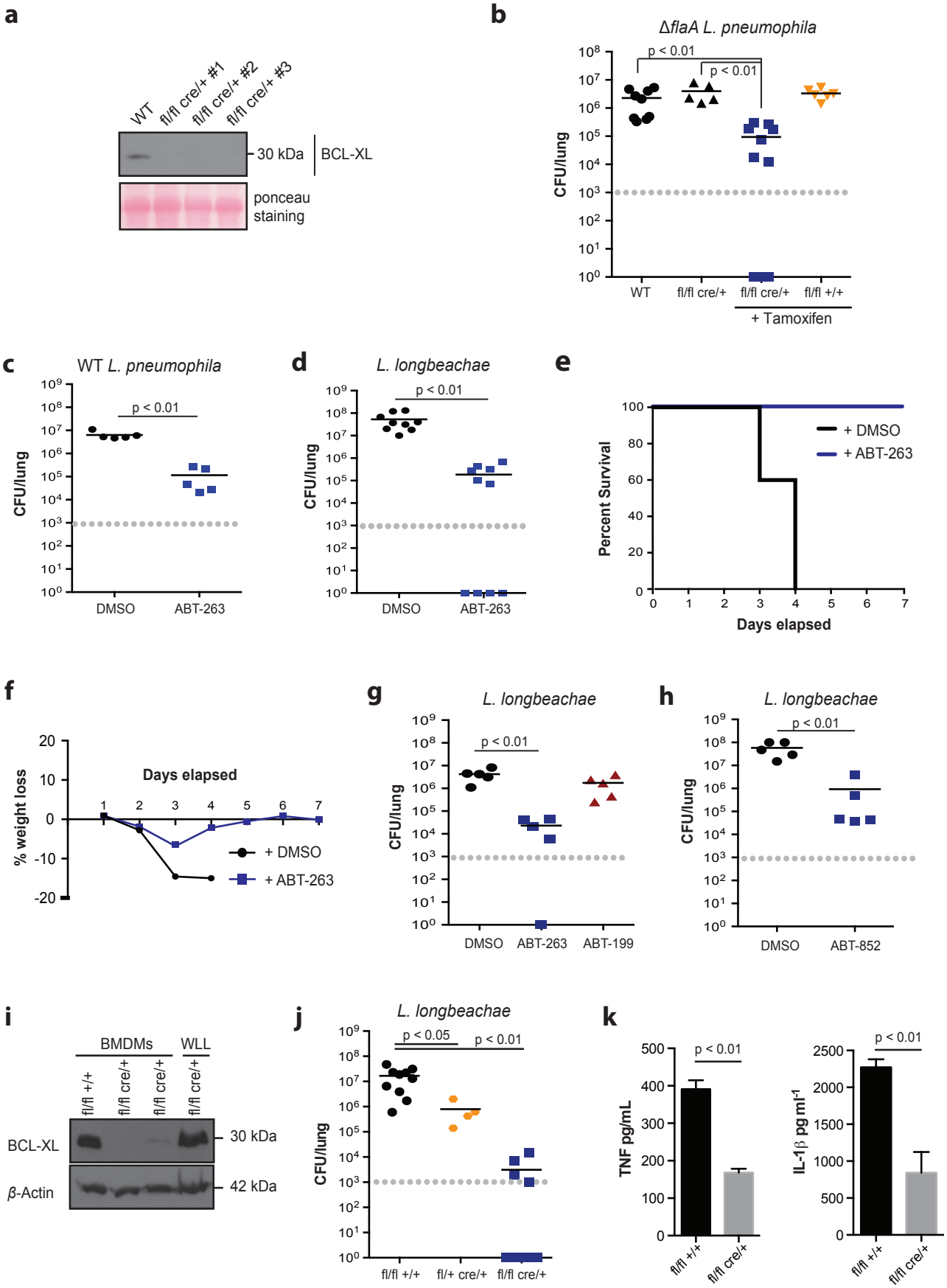
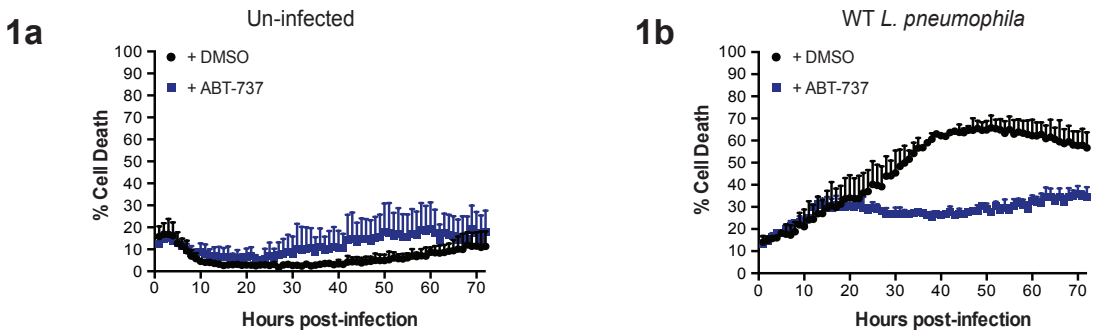
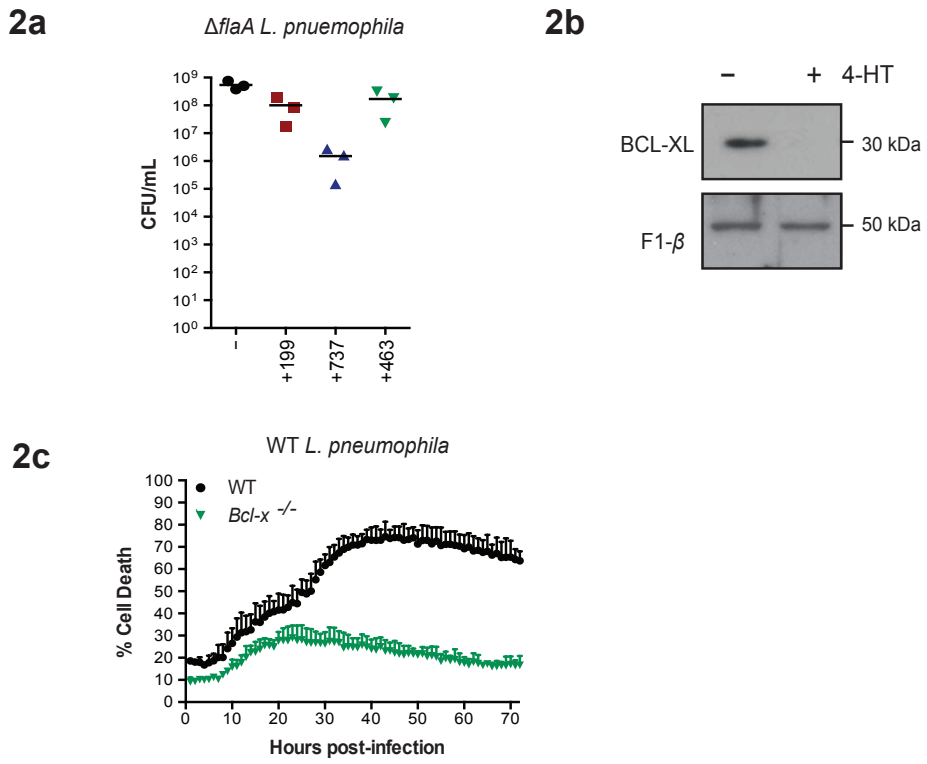

a
WT
fl/fl cre/+ #1
fl/fl cre/+ #2
fl/fl cre/+ #3
30 kDa
BCL-XL
ponceau staining
b
ΔflaA L. pneumophila
p < 0.01
p < 0.01
CFU/lung
WT
fl/fl cre/+
fl/fl cre/+
fl/fl +/+
+ Tamoxifen
c
WT L. pneumophila
p < 0.01
CFU/lung
DMSO
ABT-263
d
L. longbeachae
p < 0.01
CFU/lung
DMSO
ABT-263
e
Percent Survival
+ DMSO
+ ABT-263
Days elapsed
f
% weight loss
Days elapsed
+ DMSO
+ ABT-263
g
L. longbeachae
p < 0.01
CFU/lung
DMSO
ABT-263
ABT-199
h
L. longbeachae
p < 0.01
CFU/lung
DMSO
ABT-852
i
BMDMs
WLL
fl/fl +/+
fl/fl cre/+
fl/fl cre/+
fl/fl cre/+
BCL-XL
30 kDa
β-Actin
42 kDa
j
L. longbeachae
p < 0.05
p < 0.01
CFU/lung
fl/fl +/+
fl/+ cre/+
fl/fl cre/+
k
p < 0.01
TNF pg/mL
fl/fl +/+
fl/fl cre/+
p < 0.01
IL-1β pg ml-1
fl/fl +/+
fl/fl cre/+

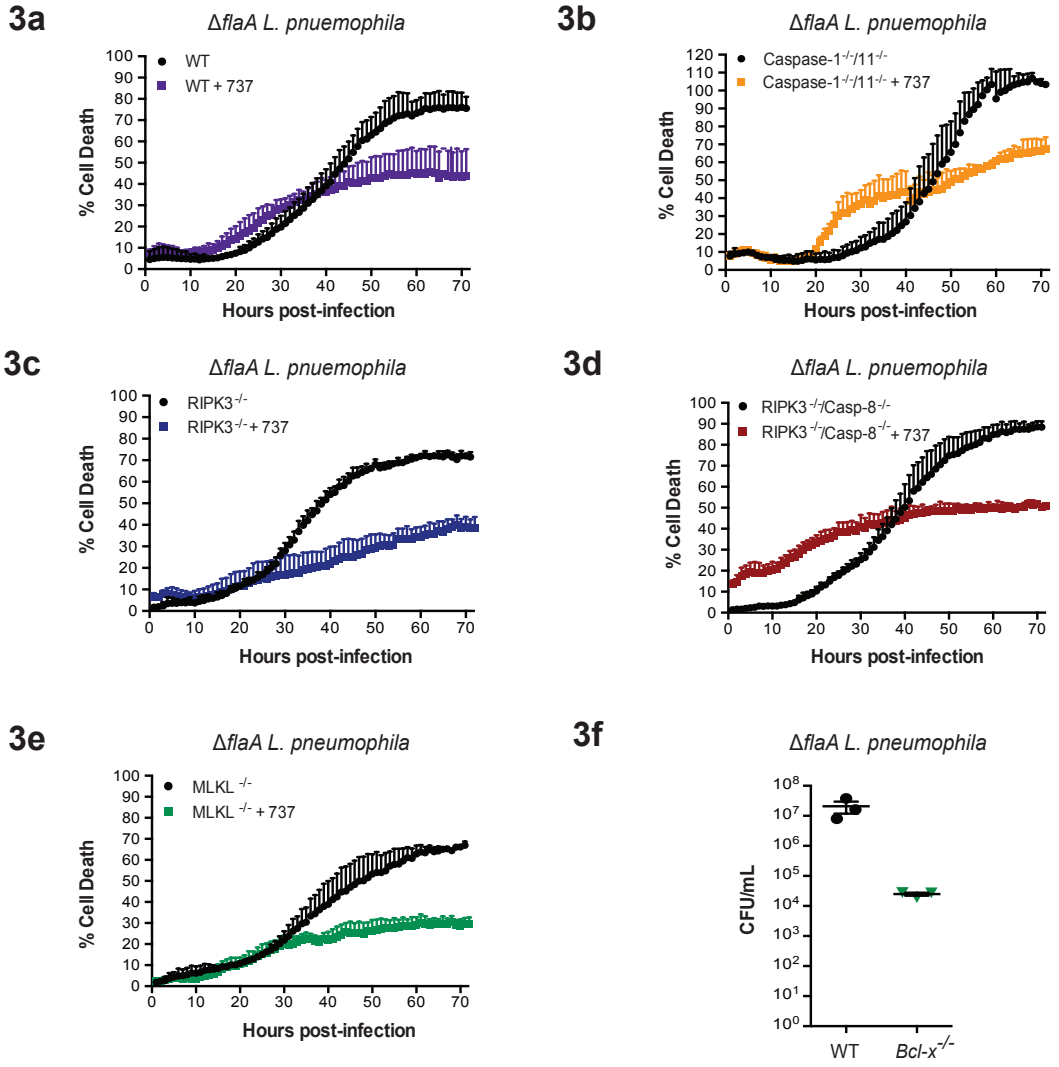
Figure 4. BCL-XL expression is required for *Legionella* replication in lungs. a, Immuno-blot analysis of BCL-XL in BMDMs derived from C57BL/6 wild-type (WT) and three *Bcl-x$^{flox/flox}$;ER-Cre* mice treated with tamoxifen. Ponceau staining as a loading control is shown. **b**, Lung bacterial burden (CFUs) of C57BL/6 and *Bcl-x$^{flox/flox}$;ER-Cre* mice 48 h after intra-nasal infection with 2.5×10^6 ΔflaA *L. pneumophila.* Mean (line) and detection limit (dotted line) are shown. **c, d,** Lung bacterial burden (CFUs) from ABT-263-treated (**c**) A/J mice after intra-nasal infection with 2.5×10^6 WT *L. pneumophila* at 48 h post-infection, or (**d**) C57BL/6 mice after intra-nasal infection with 1×10^5 *L. longbeachae* at 72 h post-infection. Mean (line) from two independent experiments and detection limit (dotted line) are shown. **e**, Mice survival curve following ABT-263 or DMSO (vehicle) treatment of *L. longbeachae*-infected C57BL/6 mice (n=5/treatment). **f**, Average body weight of each treatment group (from 4e) over the 7 days following infection (n=5/treatment). **g,** Lung bacterial burdens (CFU) from ABT-199- and ABT-263-treated C57BL/6 mice after intra-nasal infection with 1×10^5 *L. longbeachae* at 72 h post-infection. Mean (line) from two independent experiments and detection limit (dotted line) are shown. **h,** Lung bacterial burdens (CFU) from ABT-852-treated C57BL/6 mice after intra-nasal infection with 1×10^5 *L. longbeachae* at 72 h post-infection. Mean (line) from two independent experiments and detection limit (dotted line) are shown. **i,** Immuno-blot analysis of BCL-XL in whole lung lysate (WLL) from one *Bcl-x$^{flox/flox}$;LysM-Cre* mouse, and in bone marrow-derived macrophages (BMDMs) derived from one *Bcl-x$^{flox/flox}$* and two *Bcl-x$^{flox/flox}$;LysM-Cre* mice. *β*-Actin as a loading control is shown. **j,** Lung bacterial burden (CFU) of *Bcl-x$^{flox/flox}$;LysM-Cre* mice 72 h after intra-nasal infection with 1×10^5 *L. longbeachae*. Mean (line) and detection limit (dotted line) are shown. **k,** Pro-inflammatory cytokine levels in the lungs of *Bcl-x$^{flox/flox}$;LysM-Cre* and Cre-negative control mice 72 h after infection with 1×10^5 *L. longbeachae.* Mean and SE shown (n=10).



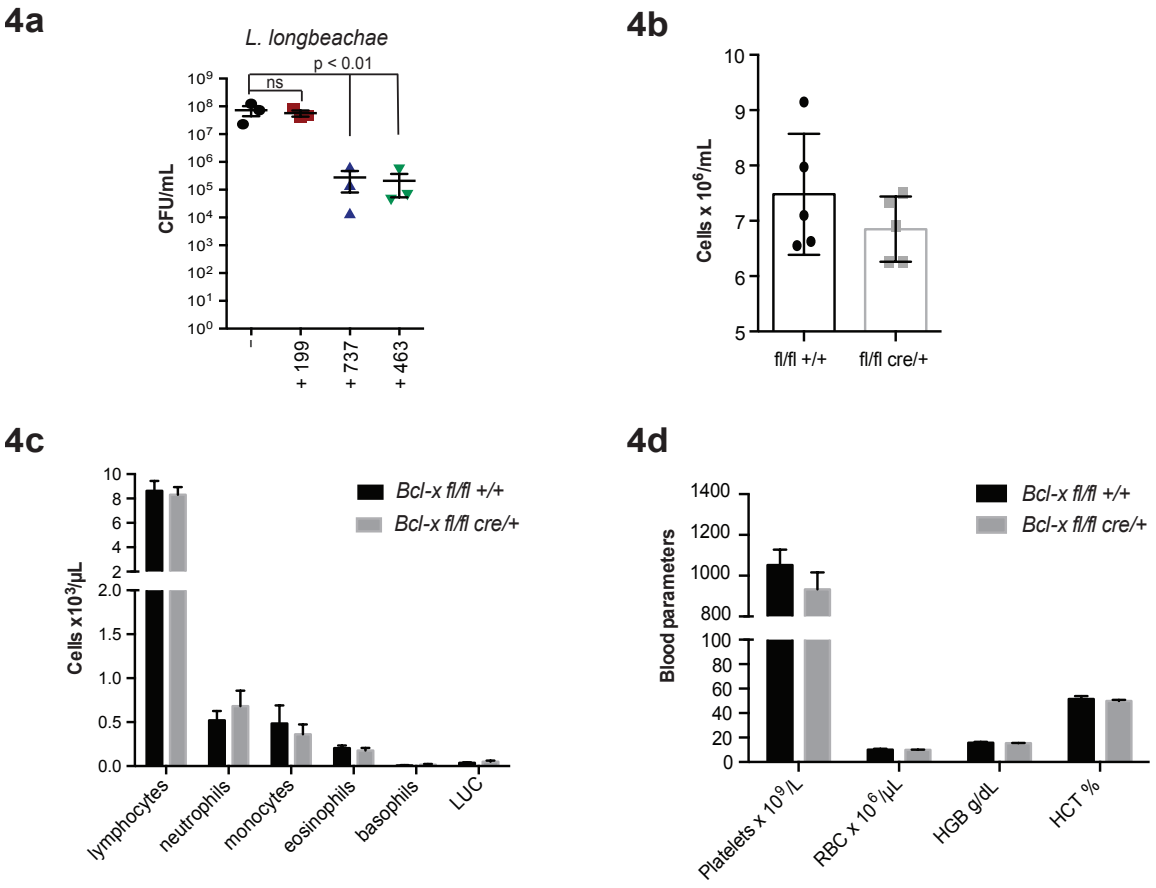
Extended Data Figure 1. a, b, Draq7-positive (dead) bone marrow-derived macrophages (BMDMs) were quantified over 72 h using time-lapse imaging in the presence of 10 μM ABT-737 or vehicle (DMSO) (**b**) without infection or (**a**) after infection with wild-type *L. pneumophila*, at an MOI of 10. Mean and SD are shown. Data representative of at least three independent experiments.



Extended Data Figure 2. a Bacterial burden (CFUs) of Δ*flaA L. pneumophila*-infected THP-1 monocyte-derived macrophages treated with 500 nM of BH3-mimetic, or control vehicle, at 48 h post-infection. Mean and SEM shown (n = 3). **b,** Immuno-blot analysis of BCL-XL protein content in bone-marrow derived macrophages (BMDMs) derived from *Bcl-x*$^{flox/flox}$*;ER-Cre* mice, whereby progenitor cells were left untreated, or else treated with 4-hydroxytamoxifen (4-HT; 50 nM, 2 days post-harvesting) to induce deletion of the two floxed *Bcl-x* alleles. **c**, Live-cell microscopic analysis of Draq7-positive (dead) WT (C57BL/6) and BCL-XL-deficient BMDMs infected with WT *L. pneumophila*. Mean and SD are shown. Data representative of at least three independent experiments.



Extended Data Figure 3. a, b, Draq7-positive (dead) **(a)** wild-type and **(b)** Caspase-$1^{-/-}$/$11^{-/-}$ bone marrow-derived macrophages (BMDMs) were quantified over 72 h using time-lapse imaging in the presence of 500 nM ABT-737 or vehicle (DMSO). **c, d, e,** Draq7-positive **(c)** $RIPK3^{-/-}$, **(d)** $RIPK3^{-/-}$/Caspase-$8^{-/-}$, and **(e)** $MLKL^{-/-}$ BMDMs were quantified over 72 h using time-lapse imaging in the presence of 500 nM ABT-737 or vehicle. **f,** Bacterial burden (CFUs) of Δ*flaA L. pneumophila* in WT (C57BL/6) and BCL-XL-deficient BMDMs isolated from tamoxifen-treated *Bcl-x$^{flox/flox}$;ER-Cre* mice, at 48 h post-infection. Mean and SE are shown (n = 3).



Extended Data Figure 4. a, Bacterial burden (CFUs) of *Legionella longbeachae*-infected BMDMs treated with 500 nM of BH3-mimetic, or control vehicle, at 48 h post-infection. Dotted line shows infection level at 6 h. Mean and SE are shown (n = 3). **b, c, d,** Phenotyping of *Bcl-x*$^{flox/flox}$*;LysM-Cre* deletion showing **(a)** BMDMs/mL derived per leg (n = 5), **(b)** naïve blood counts (cells/µL), and **(c)** blood parameters, including platelets/µL, red blood cells (RBC)/µL, % haemoglobin (HGB) and % haematocrit (HCT), indicating the proportion of total *blood* volume that is composed of RBCs. Mean and S.E.M. shown. Data representative of at least two independent experiments.

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CHAPTER 5

BCL-XL DELETION OR ANTAGONISM SENSITISES MACROPHAGES TO DEATH FOLLOWING PROTEIN SYNTHESIS INHIBITION

5.1 Introduction

The results in Chapter Four show that *Legionella*-infected macrophages depend critically upon the anti-apoptotic activity of BCL-XL, but not other BCL-2 family members, for viability. In the absence of BCL-XL, *Legionella*-infected cells undergo canonical mitochondrial apoptosis, which abolishes bacterial replication and dissemination. In mice, a single dose of BCL-XL-targeted BH3-mimetic therapy significantly reduces *Legionella* burden in the lungs and prevents lethal bacterial infection. However, the exact reason for this marked sensitivity to BCL-XL expression in *Legionella*-infected macrophages remains unknown. Thus, the primary aim of the work described in this chapter was to investigate the signalling pathways upstream of BCL-XL, in order to elucidate the mechanism underlying its critical role during *Legionella* infection.

Sustained cellular stress, including persistent microbial infection, triggers the expression and activation of pro-apoptotic BH3-only proteins, which induce apoptosis if pro-survival BCL-2 proteins, *e.g.*, BCL-2, BCL-XL, and MCL-1, become sufficiently depleted (Fig. 5.1). Depending on the specific signal, BH3-only proteins may promote BAX/BAK insertion into the mitochondrial outer membrane indirectly, as is the case with BAD and NOXA, via sequestration and inhibition of the pro-survival BCL-2 proteins. Alternatively, BH3-only proteins can also activate

BAX/BAK directly, as has been observed with BID, BIM, and PUMA (Shamas-Din *et al.*, 2013; Volkmann *et al.*, 2014). Finally, induction of apoptosis may also depend upon regulating the activity of the pro-survival BCL-2 proteins, either via transcriptional or translational regulation of expression, or by post-translation modification (Hardwick and Soane, 2013; Shamas-Din *et al.*, 2013).

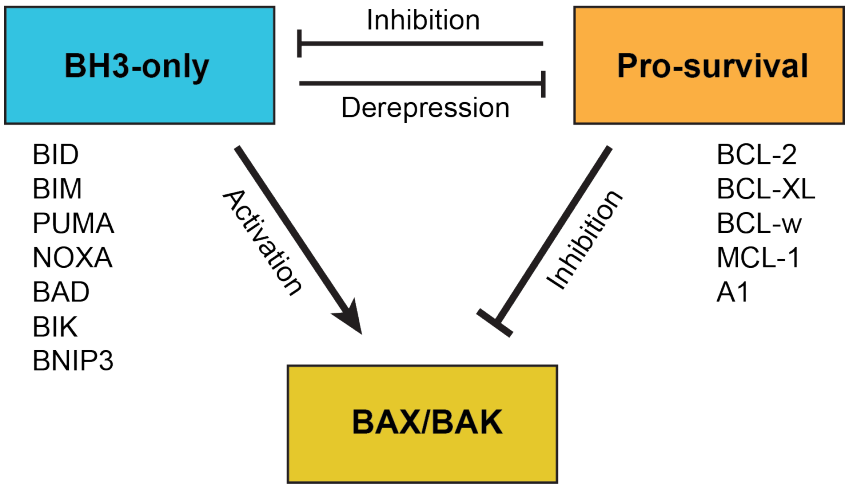


Figure 5.1 Schematic representation of the BCL-2 family interaction network. The BCL-2 protein family can be divided into three classes: (1) the pro-death proteins BAX and BAK; (2) the pro-death BH3-only proteins, of which only a subset are listed here; and (3) the pro-survival BCL-2 proteins. Figure adapted from (Westphal *et al.*, 2014).

The myriad of cellular stresses that lead to apoptosis [reviewed in Taylor *et al.*, (2008)] includes the sustained inhibition of protein synthesis by ribonucleases, antibiotics, bacterial toxins, and viruses. For example, interferon-activated RNAseL cleaves ribosomal RNA in response to viral infection, inhibiting protein synthesis and inducing apoptosis that eventually eliminates virus-infected cells (Silverman, 2003). In addition, the natural antibiotics, cycloheximide and puromycin, can target bacterial and eukaryotic cells by inhibiting protein synthesis, which, in the latter, results in the induction of mitochondrial apoptosis (Meijerman *et al.*, 1999). Induction of apoptosis

in response to protein synthesis inhibition depends on tipping the balance towards pro-apoptotic BAX and/or BAK, relative to the anti-apoptotic BCL-2 factors. In cycloheximide-treated cells, this occurs due to proteasome-mediated degradation of anti-apoptotic MCL-1, which is highly unstable with a half-life of approximately 30 minutes (Adams and Cooper, 2007). As such, loss of MCL-1 has also been shown to play a key role in apoptosis induced by adenovirus E1A expression (Cuconati *et al.*, 2003), UV irradiation (Nijhawan *et al.*, 2003), and the protein kinase inhibitor BAY 43-9006 (Rahmani *et al.*, 2005), all of which block protein synthesis.

Legionella infection also prevents host cell protein synthesis. The Type 4 secretion system (T4SS) effectors *lgt1*, *lgt2,* and *lgt3* are glycosyltransferases that directly modify and inhibit the host elongation factor eEF1A. In addition, at least four other effectors are known to inhibit host protein synthesis (Fontana *et al.*, 2011; Barry *et al.*, 2013). Furthermore, *Legionella* infection may also inhibit host protein synthesis independently of any bacterial effectors. Macrophages sense pathogenic *Legionella* containing a functional T4SS (Dot/Icm^{+}), but not those containing the avirulent Δ*dotA* mutant, and induce ubiquitination and proteasomal degradation of mTOR and its regulator Akt (Ivanov and Roy, 2013). Inhibition of mTOR signalling specifically prevents the cap-dependent initiation of translation, and this has been shown to induce a novel innate immune response against *Legionella* infection (Ivanov and Roy, 2013). Whether this response also depends on apoptotic factors, remains unknown.

5.2 Results

5.2.1 Absence of specific BH3-only proteins does not restore Legionella infection in the presence of ABT-737

Because BH3-only proteins relay pro-apoptotic signals in response to cellular stress events, four of the most common BH3-only proteins known to bind to BCL-XL (BID, BIM, PUMA, and NOXA) were tested to determine their involvement in apoptotic signalling during *Legionella* infection. For this, bacterial burdens (measured in CFU/mL) were calculated after 48 h of infection with Δ*flaA L. pneumophila* (Fig. 5.2a) or wild-type (WT) *L. longbeachae* (Fig. 5.2b) in WT C57BL/6 BMDMs and in BMDMs deficient in the pro-death BH3-only proteins, BID, BIM, PUMA, and NOXA, following treatment with the pan-Bcl2 inhibitor ABT-737 at a concentration of 500 nM.

Both Δ*flaA L. pneumophila* and WT *L. longbeachae* were able to replicate normally in BMDMs deficient in BID, BIM, PUMA, and NOXA (Fig 5.2a,b). As shown in the previous chapter, ABT-737 induced an approximately 100-fold reduction in bacterial burdens compared to untreated WT BMDMs. Similarly, ABT-737 still reduced bacteria burdens in BMDMs deficient in BID, BIM, PUMA, and NOXA (Fig 5.2a, b Thus, the data show that the absence of any one of these four BH3-only proteins is not sufficient to rescue *Legionella* infection from ABT-737-mediated inhibition of BCL-XL, implying that BIM, BID, PUMA and NOXA are not individually responsible for relaying the pro-apoptotic signal upstream of BCL-XL.

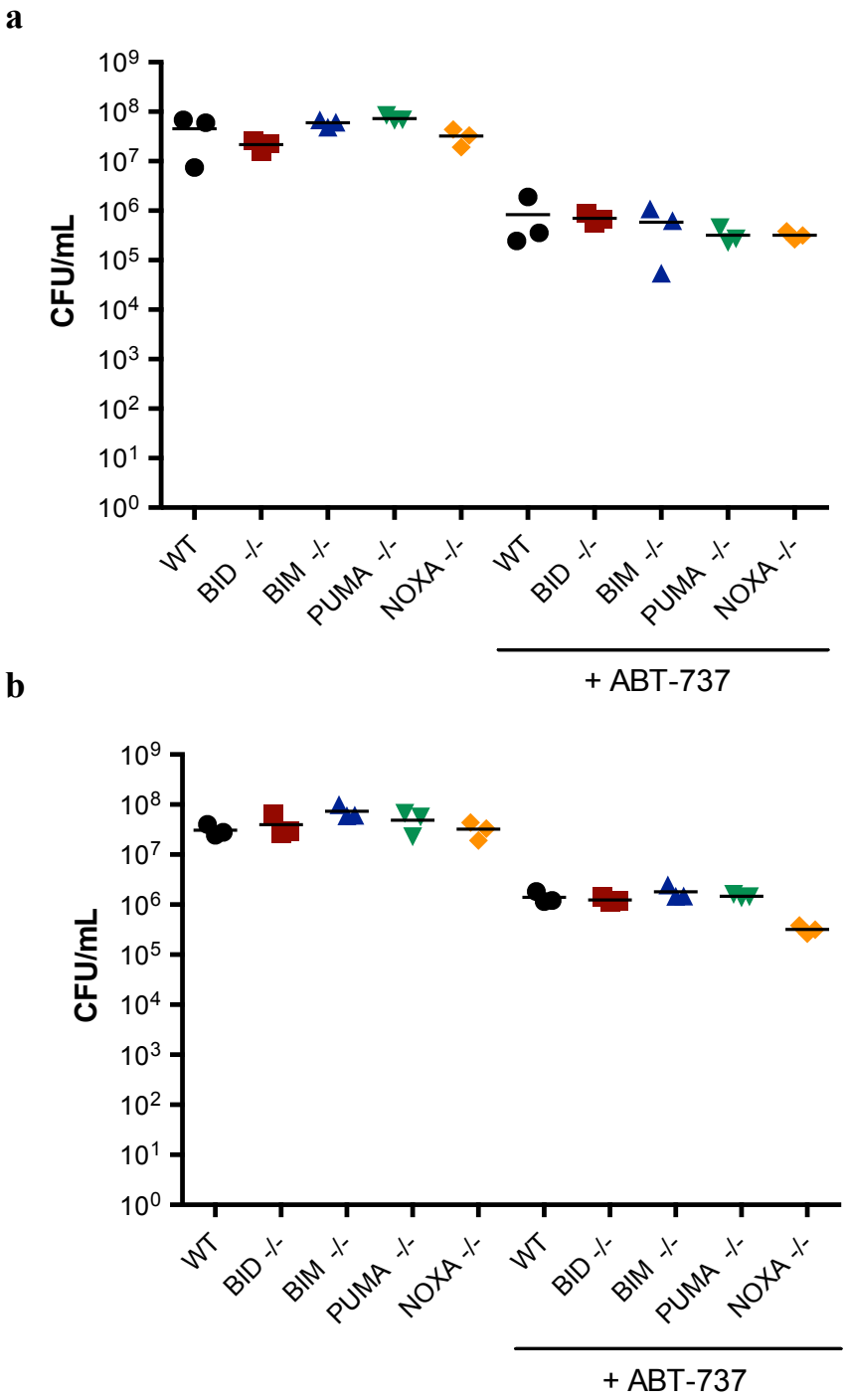


Fig. 5.2 Absence of specific BH3-only proteins does not restore *Legionella* infection in the presence of ABT-737 a, b) Bacterial burdens at 48 h post-infection, shown in colony-forming units (CFU/mL), from C57BL/6 wild-type (WT), *BID*$^{-/-}$, *BIM*$^{-/-}$, *PUMA*$^{-/-}$, and *NOXA*$^{-/-}$ BMDMs treated with 500 nM ABT-737, or left untreated, before infection with **(a)** Δ*flaA L. pneumophila* or **(b)** WT *L. longbeachae* (MOI = 50, 2 h). Mean shown of three independent experiments shown.

5.2.2 Pro-death BAK has a minor role in apoptosis induced by loss of BCL-XL

Have established that deletion of both the pro-death BCL-2 proteins BAX and BAK blocks ABT-737-mediated cell death (Chapter 4, Fig 4.3), the involvement of BAK, in particular, was tested, as BAK is a common binding partner for BCL-XL (Rooswinkel *et al.*, 2014). Live-cell imaging was used to quantify Draq7-uptake in WT C57BL/6 and $BAK^{-/-}$ BMDMs infected with Δ*flaA L. pneumophila* at a MOI of 10 and treated with ABT-199, ABT-737, and A-1155463 (463) at a concentration of 500 nM (Fig. 5.3a, b). In addition, bacterial burdens (measured in CFU/mL) were measured after 48 h of infection (Fig. 5.3c).

As expected, the Δ*flaA* strain induced cell death normally in the $BAK^{-/-}$ BMDMs. However, in both the WT and the $BAK^{-/-}$ BMDMs, treatment with BCL-XL-targeting ABT-737 or A-115463 resulted in approximately 20 % fewer Draq7-positive cells than in the DMSO- or BCL-2-targeting ABT-199-treated cells after 72 h of infection. In contrast, BAX/BAK double deletion completely ablates sensitivity to BCL-XL-targeting BH3-mimetics (Chapter 4, Fig 4.3). The Δ*flaA* strain also replicated approximately 10-fold less in the ABT-737- and A-115463-treated $BAK^{-/-}$ BMDMs, compared with in the untreated or ABT-199-treated BMDMs. Although this replication defect was not as pronounced as the 100-fold difference seen in the WT BMDMs, it was not the complete rescue observed in the BAX/BAK double knock-outs. Together, these data indicate that BAK by itself does not play a major role in initiating mitochondrial permeabilisation following loss of BCL-XL in *Legionella*-infected cells.

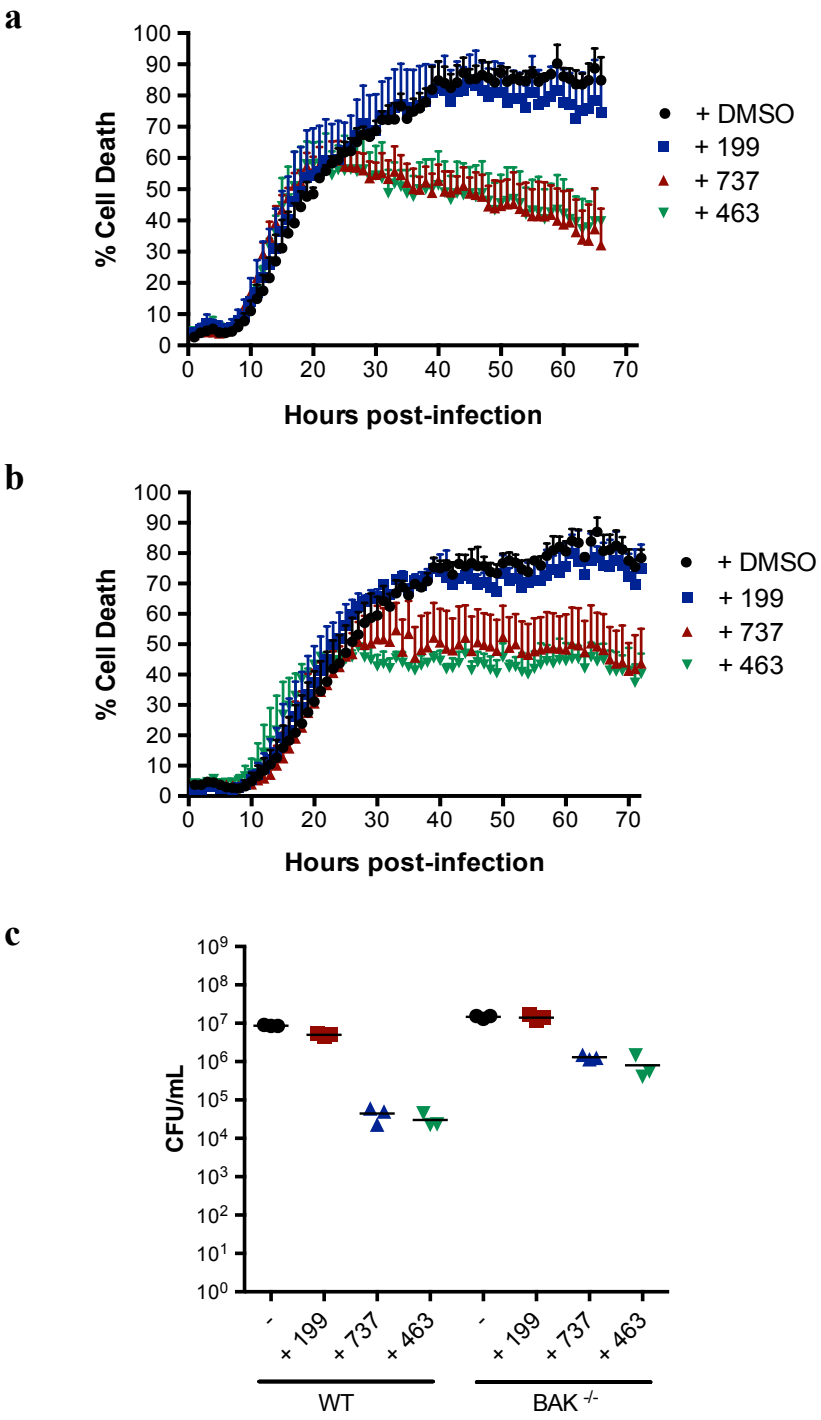


Figure 5.3 Apoptosis induced by loss of BCL-XL proceeds via BAX, not BAK a, b) Draq7 positive **(a)** WT C57BL/6 and **(b)** BAK$^{-/-}$ BMDMs were quantified by live-cell imaging over 72 h, following infection with Δ*flaA L. pneumophila* at a MOI of 10, and addition of ABT-199, ABT-737, and A-1155463 (463) [500 nM]. Data are representative of three independent experiments. Mean and S.D. of three independent biological replicates shown. **(c)** Bacterial burdens at 48 h post-infection, shown in colony-forming units (CFU/mL), from C57BL/6 wild-type and BAK-/- mice infected at a MOI of 10 with ΔflaA L. pneumophila and treated with ABT-199, ABT-737, or A-1155463(463) [500 nM]. Mean of three independent experiments shown.

5.2.3 Anti-apoptotic MCL-1 is depleted during Legionella infection

The interaction between the pro-survival and pro-death BCL-2 proteins is thought to involve a series of competing equilibria, in which the relative concentrations of available binding partners determine whether the cell lives or dies (Shamas-Din *et al.*, 2013). Thus, immunoblotting analysis was used to determine whether the key anti-apoptotic BCL-2 proteins, BCL-2, MCL-1, and BCL-XL were up- or down-regulated during *L. pneumophila* infection of BMDMs (Fig. 5.4). To ensure that a large proportion of BMDMs was infected, a high MOI of 50 coupled with centrifugation, was used.

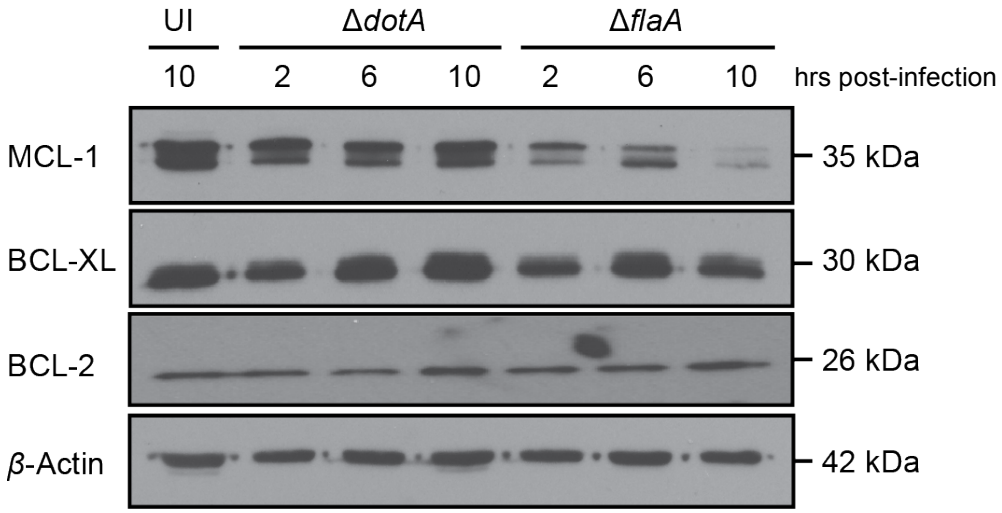


Figure 5.4 Anti-apoptotic MCL-1 is depleted during *Legionella* infection Time course immuno-blot analysis for BCL-XL, BCL-2, and MCL-1 in wild type (WT) bone marrow-derived macrophages (BMDMs) left uninfected (UI), or infected at an MOI of 50 with Δ*flaA* or Δ*dotA L. pneumophila*. *β*-actin blot is a loading control. Blot is representative of two independent experiments.

The immuno-blot for *β*-actin shows consistent protein loading across all the wells. Both BCL-2 and BCL-XL levels remained stable over the first 10 h of infection with both Δ*dotA* and Δ*flaA L. pneumophila.* In contrast, after 2 h of infection, the level of

MCL-1 was already substantially reduced in Δ*flaA*-infected BMDMs, relative to that seen in the uninfected, or Δ*dotA*-infected, cells. Furthermore, by 10 h, the level of MCL-1 dropped by more than 75 % compared with its level at 2 h, indicating that depletion of MCL-1 occurs early during Dot/Icm$^+$ *Legionella* infection. This result suggests that *Legionella*-infected cells may become sensitised to BCL-XL antagonism due to a reduction in MCL-1 levels.

5.2.4 Stabilisation of MCL-1 limits ABT-737-mediated cell death in the presence of cycloheximide

The protein synthesis inhibitor cycloheximide (CHX) is thought to trigger apoptosis primarily because of the short half-life of MCL-1 and its rapid depletion in the absence of new protein synthesis (Adams and Cooper, 2007). To initially test whether CHX treatment also sensitises BMDMs to cell death following BCL-XL antagonism, BMDMs were treated with CHX in the presence of ABT-737, ABT-199, or A-1155463 (Fig 5.5). Similar to *Legionella* infection, CHX-treated BMDMs remained viable (PI-negative) for up to 6 h, but showed increased cell death rates in the presence of ABT-737 and A-1155463 (~ 40 % and 50 %, respectively), but not ABT-199. As expected, the pan-caspase inhibitor Q-VD prevented ABT-737/CHX-induced cell death. To verify that CHX/ABT-737-induced cell death is largely due to the loss of MCL-1, CHX treatment was repeated with BMDMs expressing a stabilised form of MCL-1, termed MCL1-fN, due to an extended N-terminus that prevents proteasomal degradation to some degree, extending its half-life to 2 – 3 h (Rooswinkel *et al.*, 2014). In contrast to WT BMDMs, the CHX/ABT-737- and CHX/A-1155463-treated MCL-1-fN BMDMs were almost all PI-negative, similar to the WT BMDMs treated with CHX alone or in combination with ABT-199, which does not target BCL-XL. Thus,

stabilising MCL-1 prevents apoptotic cell death induced by CHX treatment, even in the absence of BCL-XL activity.

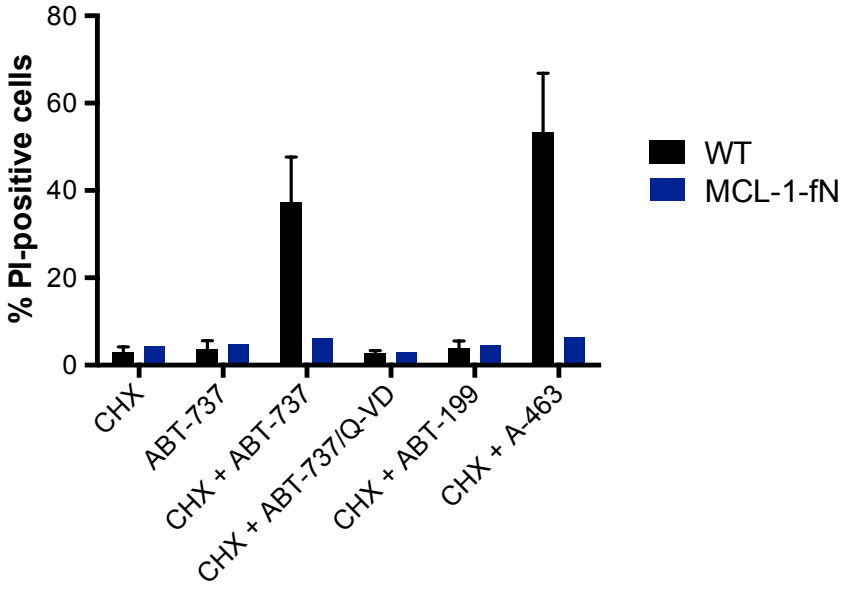


Figure 5.5 Stabilisation of MCL-1 prevents ABT-737-mediated cell death in the presence of cycloheximide Propidium iodide (PI)-positive WT C57BL/6 BMDMs and BMDMs with a stabilised MCL-1 protein (MCL-1-fN) were quantified by FACS after 6 h of treatment with cycloheximide (CHX) alone, or in combination with, the BH3 mimetics ABT-199, ABT-737, and A-1155463 (463) [500 nM]. Q-VD was used at 20 μM. Mean and S.E.M. of two independent experiments shown.

5.2.5 Stabilisation of MCL-1 limits ABT-737-mediated cell death during Legionella infection

To determine if stabilising MCL-1 could also rescue the ABT-737 phenotype following *Legionella* infection, WT and MCL-1-fN BMDMS were infected at a MOI of 50 with Δ*dotA* or Δ*flaA L. pneumophila* and treated with ABT-737 at a concentration of 500 nM, for 6 h. Cell viability was determined by flow cytometry and PI uptake (Fig. 5.6). Similar to CHX treatment, Dot/Icm$^+$ *Legionella* infection, followed by ABT-737 treatment, induced approximately 20 % less cell death in the MCL-1-fN BMDMs when compared to WT BMDMs. In contrast, the MCL-1-fN BMDMs showed more cell death following Δ*dotA* infection, irrespective of ABT-737

treatment. These data suggest that stabilising MCL-1 during *Legionella* infection prevents ABT-737-mediated apoptotic cell death and support the idea that *Legionella*-infected cells die following BCL-XL antagonism because of reduced MCL-1 levels.

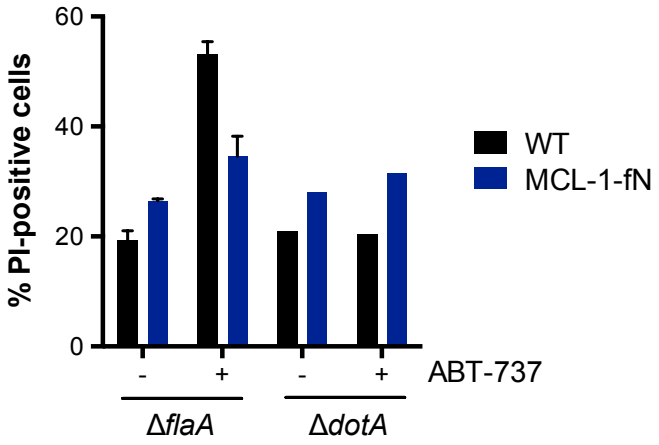


Figure 5.6 Stabilisation of MCL-1 limits ABT-737-mediated cell death during *Legionella* infection PI-positive WT C57BL/6 BMDMs and BMDMs with a stabilised MCL-1 protein (MCL-1-fN) were quantified by FACS after 6 h of infection with Δ*dotA* and Δ*flaA L. pneumophila* and treatment with 500 nM of ABT-737. Mean and S.E.M. of two independent experiments shown.

5.2.6 BCL-XL limits apoptosis induced by the inhibition of protein synthesis

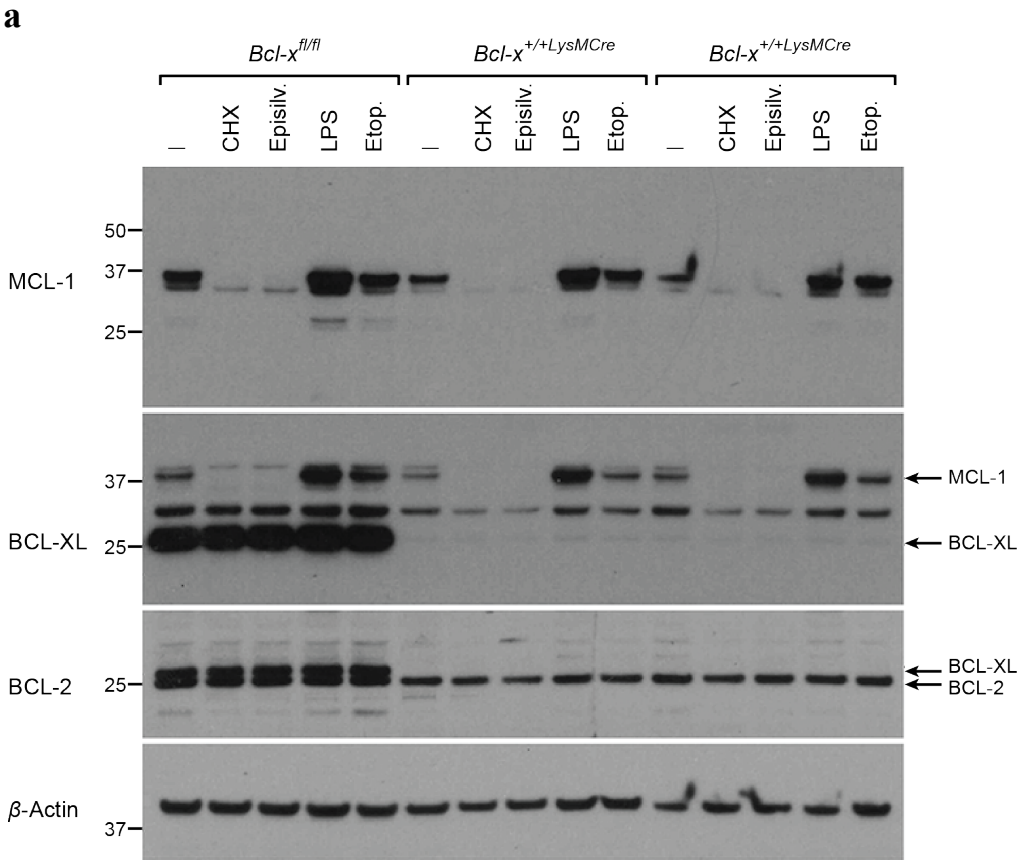
To confirm that BCL-XL expression is required for preventing rapid cell death following the inhibition of protein synthesis, and that this is a consequence of reduced MCL-1 levels, BMDMs-derived from *Bcl-x*$^{flox/flox}$*;LysM-Cre* and *Bcl-x*$^{flox/flox}$ mice were treated with the protein synthesis inhibitors cyclohexamide (CHX) and episilvestrol (EPI) either alone, or in combination with, lipopolysaccharide (LPS) to mimic bacterial infection (Fig. 5.7a, b). BMDMs were also treated with the DNA topoisomerase II inhibitor, etoposide, which was used as a positive control for the induction of apoptosis. Western blotting showed that MCL-1 was degraded in both the *Bcl-x*$^{flox/flox}$ and *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs after 5 h of treatment with

CHX and EPI (Fig. 5.7a). In comparison, BCL-2 levels remained stable, as did BCL-XL levels in the *Bcl-x*$^{flox/flox}$-derived BMDMS. Furthermore, MCL-1 did not disappear upon treatment with etoposide, demonstrating that induction of apoptosis does not necessarily depend upon MCL-1 degradation.

After 5 h, less that 20 % of *Bcl-x*$^{flox/flox}$ BMDMs treated with cycloheximide or episilvestrol were propidium iodide (PI)-positive (*i.e.*, dead), similar to the number of untreated cells (Fig. 5.7b). Furthermore, the addition of LPS alone, or in combination with CHX and EPI, did not significantly increase the number of PI-positive cells. In contrast, 60 – 80 % of the CHX- and EPI-treated *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs were PI-positive by this time, irrespective of LPS addition. This was also significantly more that the number of PI-positive etoposide-treated *Bcl-x*$^{flox/flox}$ or *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs, indicating that BCL-XL-deficient cells are not simply over-sensitive to all pro-apoptotic stimuli. After 22 h, approximately 50 % of CHX- and EPI-treated *Bcl-x*$^{flox/flox}$ BMDMs were PI-positive, whereas close to 90 % of *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs were PI-positive. Although close to 50 % of the etoposide-treated *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived BMDMs were also PI-positive at this time, compared to fewer that 20 % of *Bcl-x*$^{flox/flox}$ BMDMs, this was still significantly fewer than the number of CHX- or EPI-treated *Bcl-x*$^{flox/flox}$*;LysM-Cre*-derived PI-positive BMDMs.

To further confirm that BCL-XL expression is essential for optimal cellular viability following the inhibition of protein synthesis, the percentage of dead WT BMDMs was quantified, either by live-cell imaging or flow cytometry, following treatment with ABT-199, ABT-737, or A-1155463, and the *Pseudomonas aeruginosa* exotoxin A (ExoA), which is a potent inhibitor of the elongation factor eEF2 (Yates and Merrill,

2004) (Fig. 5.7c, d). Cell death in the BMDMs treated with both ExoA and ABT-737 was more rapid that in the BMDMs treated with ExoA alone, suggesting that these cells undergo apoptosis earlier when BCL-XL activity is inhibited. In addition, after 24 h, over 30 % of ExoA- and A-1155463-treated BMDMs were propidium iodide (PI)-positive, compared to less than 5 % of ExoA- or ExoA/ABT-199-treated BMDMs. The fact that co-treatment with ABT-737 only resulted in approximately 10 % of cells being PI-positive after 24 h merely indicates that it is less effective at targeting BCL-XL than other BCL-2 proteins, *e.g.*, BCL-2, as reported in (Rooswinkel *et al.*, 2012). Together, these data indicate that chemical BCL-XL antagonism, or its genetic deletion, sensitises cells to apoptosis following the inhibition of protein synthesis by cycloheximide, episilvestrol, or exotoxin A.



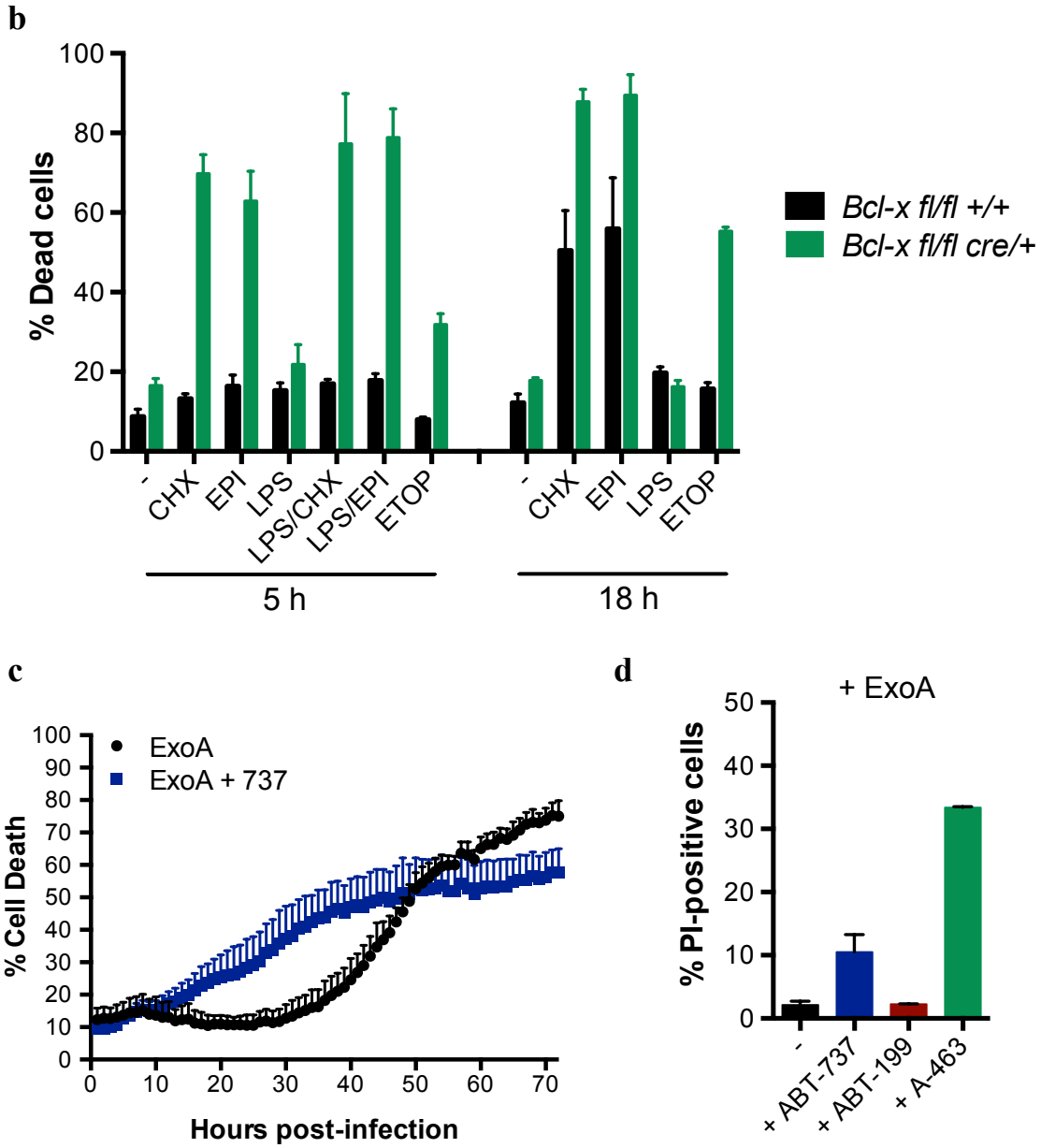


Figure 5.7 BCL-XL expression limits apoptosis caused by the inhibition of protein synthesis a) Immuno-blot analysis for *Bcl-x*$^{flox/flox}$ and *Bcl-x*$^{flox/flox}$*;LysM-Cre* BMDMs treated with cycloheximide (CHX), episilvestrol (EPI), LPS, and etoposide for 5 h. *β*-actin blot is a loading control. Blot is representative of at three independent experiments. **(b)** Propidium iodide (PI)-positive *Bcl-x*$^{flox/flox}$ and *Bcl-x*$^{flox/flox}$*;LysM-Cre* BMDMs were quantified by FACS after 5 or 18 h of treatment with the protein synthesis inhibitors cycloheximide (CHX) and episilvestrol (EPI) alone, or in combination with, lipopolysaccharide (LPS). Etoposide was used as a positive control for apoptosis induction. Mean and S.E.M. of three independent experiments shown. **(c)** Draq7-positive WT C57BL/6 BMDMs were quantified by live-cell imaging over 72 h, following treatment with 500 ng/mL Exotoxin A (ExoA) and ABT-737 [500 nM]. Mean + S.D. shown. Data are representative of three independent experiments **(d)** Propidium iodide positive WT C57BL/6 BMDMs were quantified by FACS after 24 h of treatment with ExoA and ABT-199, ABT-737, and A-1155463. Data are representative of two independent experiments. Mean and S.D. of three independent biological replicates shown.

5.2.7 Legionella infection partially inhibits host cell protein synthesis

To investigate whether the inhibition of host protein synthesis during *Legionella* infection was also responsible for sensitising cells to apoptosis following BCL-XL antagonism, it was first necessary to confirm that *L. pneumophila* 130b inhibits host protein synthesis, as has been reported with other *L. pneumophila* serogroups (Fontana *et al.*, 2011; Ivanov and Roy, 2013). C57BL/6 WT BMDMs were infected with Δ*dotA* or Δ*flaA L. pneumophila*, or with a pathogenic, non-replicating *L. pneumophila* strain that possesses a functional Type IV secretion system, but lacks the ability to translocate effector proteins (Δ*icmS*), at a high multiplicity of infection (MOI) of 50, coupled with centrifugation. Protein synthesis was studied by radio-labelling of proteins with [^{35}S] methionine before SDS-PAGE analysis after 6 or 18 h of infection (Fig. 5.8).

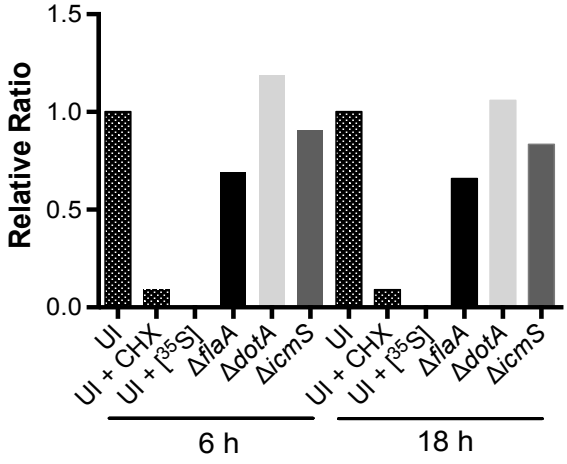


Figure 5.8 *Legionella* infection partially inhibits host cell protein synthesis Relative ratio of radio-labelled proteins in WT C57BL/6 BMDMs treated with cycloheximide (CHX) [1 μg.mL^{-1}] or infected at an MOI of 50 with Δ*flaA*, Δ*dotA*, and Δ*icmS L. pneumophila* at 6 and 18 h post-infection. Mean shown. Data from one experiment of two shown.

After 6 h, protein synthesis had decreased by over 90 % in the BMDMs treated with cycloheximide (CHX). Although not as marked as the CHX-treatment, at both 6 and

18 h post-infection, protein synthesis was reduced by approximately 35 % in BMDMs infected with Δ*flaA L. pneumophila*, relative to uninfected BMDMs. In comparison, protein synthesis increased in BMDMs infected with Δ*dotA L. pneumophila*. Host protein synthesis in the Δ*icmS*-infected BMDMs showed an intermediate phenotype whereby protein levels were reduced, likely due to down-regulation of mTOR, which occurs independently of T4SS-translocation (Ivanov and Roy, 2013), but not to the extent seen in Δ*flaA*-infected BMDMs. Thus, these data demonstrate that pathogenic Dot/Icm$^+$ *L. pneumophila* 130b partially inhibits host cell protein synthesis within 6 h of infection.

5.2.9 The requirement for BCL-XL expression in Legionella infection is effector mediated.

Inhibition of host protein synthesis during *Legionella* infection occurs via both effector-dependent and effector-independent mechanisms [reviewed in Rolando and Buchrieser, (2014)]. To determine whether infected cells become sensitised to BCL-XL expression merely upon detection of pathogenic *Legionella*, or whether the requirement for BCL-XL is effector-mediated, live-cell imaging was used to quantify cell death over 72 h in Draq7-stained C57BL/6 wild-type (WT) bone marrow-derived macrophages (BMDMs) treated with ABT-737 and infected with the Δ*icmS L. pneumophila* strain (Fig 5.9a). To ensure that a large percentage of cells was initially infected, a high MOI of 25 coupled with centrifugation, was used. If the requirement for BCL-XL is effector-mediated then BMDMs infected with the Δ*icmS* strain should remain Draq7-negative following ABT-737 treatment.

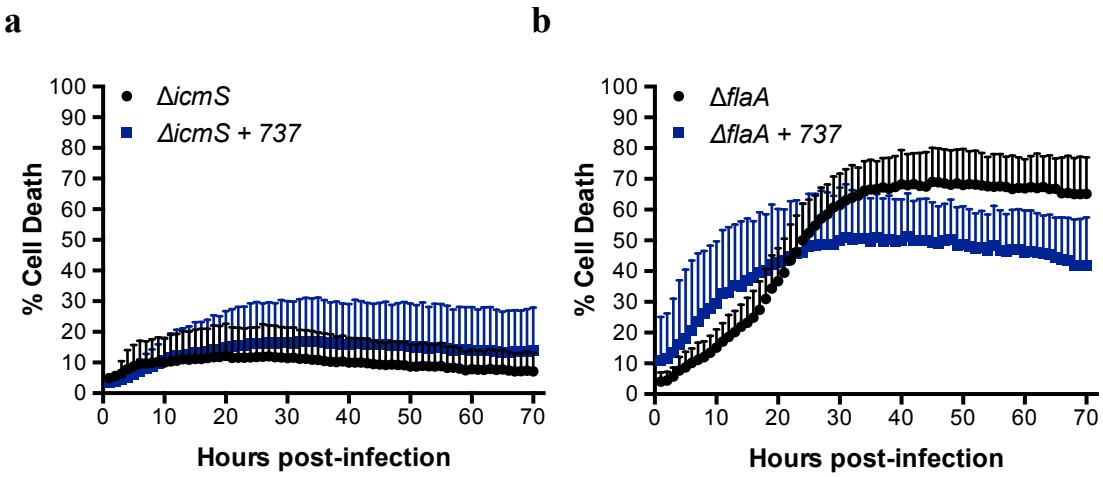


Figure 5.9 The requirement for BCL-XL in *Legionella* infection is effector mediated a, b) Draq7 positive WT C57BL/6 BMDMs were quantified by live-cell imaging over 72 h, following infection with (**a**) Δ*icmS* or (**b**) Δ*flaA L. pneumophila* at a MOI of 25 + centrifugation, and addition of ABT-737 [500 nM]. Data are representative of at least three independent experiments. Mean and S.D. of three independent biological repeats shown.

To determine the initial infection level, BMDMs were infected with Δ*flaA L. pneumophila*, following treatment with ABT-737 (Fig. 5.9b) After 24 h of infection, approximately 50 % of the BMDMs infected with Δ*flaA L. pneumophila* were dead, irrespective of ABT-737 treatment. However, by 48 h post-infection the percentage of dead ABT-737-treated BMDMs had not increased above 50 %, whereas approximately 70 % of untreated BMDMs were Draq7-positive by this time, implying that the initial infection rate was around 50 %. Despite having a functional T4SS, Δ*icmS* is unable to replicate in BMDMs (similar to the Δ*dotA* strain) and, thus, is unable to induce macrophage death. Infection with the Δ*icmS* strain did not sensitise BMDMs to ABT-737-treatment, even after 72 h. This result suggests that *Legionella*-infected cells become sensitised to BCL-XL expression in an effector-dependent manner.

5.2.9 Loss of seven effectors that target host protein translation machinery does not ablate sensitivity to BCL-XL expression

To further determine whether deliberate targeting of host translational machinery by *Legionella*-translocated effectors was responsible for sensitising infected cells to death following BCL-XL antagonism, live-cell imaging was used to quantify cell death in WT C57BL/6 BMDMs that were infected at a MOI of 10 with Δ*flaA L. pneumophila* (Lp102) and a flagellin-deficient *L. pneumophila* (Lp102) strain lacking seven (Δ*flaA*/Δ*7*) of the effectors known to target the host cell translational machinery, *lgt1*, *lgt2*, *lgt3*, *sidI*, *sidL*, *ceg6*, and *ravX* (Fontana *et al.*, 2011; Barry *et al.*, 2013), after treatment with ABT-737 at a concentration of 500 nM (Fig. 5.10a, b).

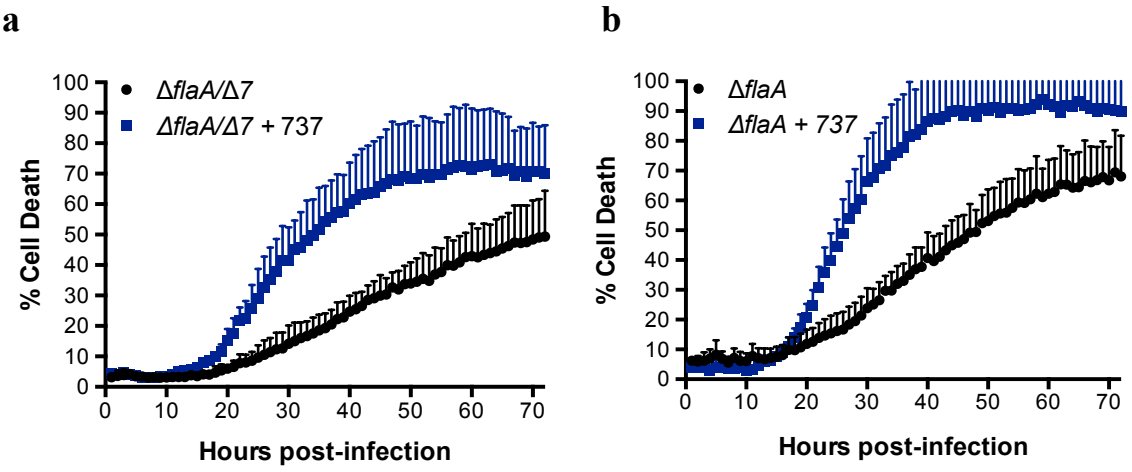


Figure 5.10 Loss of seven effectors that target host protein translation machinery does not ablate sensitivity to BCL-XL expression a, b) Draq7 positive WT C57BL/6 BMDMs were quantified by live-cell imaging over 72 h, following infection with **(a)** Δ*flaA*/Δ*7* or **(b)** Δ*flaA L. pneumophila* (Philadelphia Lp102) at a MOI of 10 and addition of ABT-737 [500 nM]. Mean and S.D. from three biological repeats from one independent experiment shown.

Treatment with ABT-737 induced rapid cell death in BMDMs infected with the Δ*flaA* strain, despite the relatively low MOI of 10, likely reflecting an increased rate of infection with the *L. pneumophila* Philadelphia serogroup. The difference between the treated and untreated cells was most pronounced at 40 h post-infection, with approximately 40 % more Draq7-positive ABT-737-treated cells than untreated.

Despite the deletion of seven effectors, Δ*flaA*/Δ*7* mutant was also able to kill BMDMs at a similar rate to the Δ*flaA* strain. Unexpectedly, ABT-737 still induced rapid cell death in macrophages infected with the Δ*flaA*/Δ*7* mutant, similar to that seen in the Δ*flaA*-infected macrophages. Consequently, loss of seven effectors known to inhibit host protein synthesis during *Legionella* infection does not ablate the sensitivity of infected macrophages to BCL-XL expression.

5.3 Discussion

In this chapter I have identified the mechanism that sensitises *Legionella*-infected macrophages to the anti-apoptotic activity of BCL-XL. *Legionella* inhibit host protein synthesis early during infection, and this leads to rapid loss of the highly unstable pro-survival BCL-2 protein, MCL-1. Accordingly, MCL-1 is also rapidly depleted in cells treated with protein synthesis inhibitors, *e.g.*, cycloheximide or *P. aeruginosa* Exotoxin A. In the absence of BCL-XL activity, either due to genetic deletion or pharmacological inhibition, inhibition of protein synthesis leads to rapid apoptosis mediated by BAK and BAX, which can be blocked by stabilising MCL-1. Thus, loss of MCL-1 is the key factor that sensitises *Legionella*-infected cells to death following BCL-XL antagonism.

While BCL-XL can interact with both pro-death BAX and BAK, MCL-1 primarily binds BAK to prevent mitochondrial apoptosis (Fletcher *et al.*, 2008; Zhai *et al.*, 2008; Rooswinkel *et al.*, 2014). Consequently, apoptosis resulting from inhibition of protein synthesis by silvestrol is prevented in the absence of BAK, at least in some cell lines (Lindqvist *et al.*, 2012). Surprisingly, therefore, while BAK-deficient BMDMs rescued *Legionella* growth to some extent in the presence of ABT-737, they failed to prevent ABT-737-mediated killing of *Legionella*-infected macrophages to the same degree as BAK/BAX double-deficient BMDMs. This suggests a crucial role for BAX or, alternatively, that BAX and BAK may work in concert to initiate apoptosis. It is likely that cells become sensitised to BAK activation following loss of MCL-1 in *Legionella*-infected macrophages. BCL-XL, which is able to bind both BAX and BAK, thus becomes the critical regulator of apoptosis. In contrast, BCL-2 does not bind well to BAK (Willis *et al.*, 2005; Rooswinkel *et al.*, 2014), and failure of BCL-2-targeting

BH3-mimetics, *e.g.*, ABT-199, to induce apoptosis in *Legionella*-infected macrophages further supports the notion that both BAX and BAK can initiate apoptosis.

Particularly at low infection levels, cell death of BCL-XL-deficient BMDMs is only detectable in the late stages of *Legionella* infection (~20 h post-infection). This suggests either that the block in protein synthesis induced by *Legionella* is not sufficient to trigger complete loss of MCL-1 or, alternatively, that BAK and BAX remain inactive, despite MCL-1 depletion. Although, when freed from constraint by pro-survival factors, BAX can initiate apoptosis without an additional stimulus (Fletcher *et al.*, 2008), in general, BAX/BAK-mediated apoptosis depends upon a second signal that triggers BH3 domain exposure and pore-complex formation. Thus, *Legionella*-infected cells may become sensitised to BCL-XL antagonism in two temporally distinct stages (Fig. 5.11).

The first stage involves loss of MCL-1 due to protein synthesis inhibition. This is evident at high infection rates, whereby a stabilised form of MCL-1 is able to prevent rapid ABT-737-induced cell death in Δ*flaA*-infected macrophages. In the second stage, infected cells become 'primed' for death, whereby they rely on BCL-XL expression to hold BAX/BAK activity in check, either directly, or via inhibition of another pro-death BH3-only protein. Activation of a BH3-only protein is possible because, in contrast to cycloheximide treatment, *Legionella* infection does not completely inhibit host protein synthesis, allowing certain transcripts to bypass the translational block (Fontana *et al.*, 2011; Asrat *et al.*, 2014). The nature of the 'priming' signal is unknown, but may simply represent a stress response to on-going infection and the hijacking of host cell pathways by bacterial effectors. The failure of ABT-737 to induce cell death in the

Δ*icmS*-infected cells, despite down-regulation of mTOR (Ivanov and Roy, 2013), supports this idea, indicating that cells do not become sufficiently stressed without bacterial replication and effector translocation.

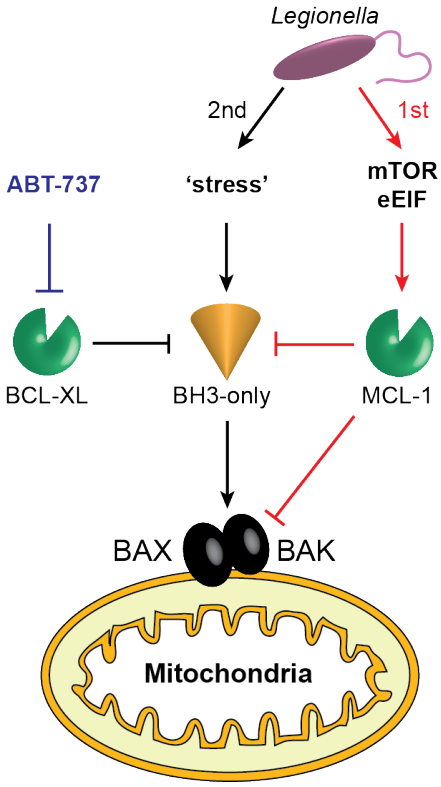


Figure 5.11 *Legionella*-infected cells may become sensitised to BCL-XL in two stages. In the first stage (red arrows), *Legionella* inhibit host protein synthesis, resulting in loss of MCL-1. In the second stage (black arrows), *Legionella* activate BAX/BAK directly, or via an unknown BH3-only protein(s). BCL-XL inhibits BAX and BAK until it is sequestered by ABT-737, resulting in cell death.

MCL-1 and BCL-XL have quite different binding partners among the BH3-only proteins, with the exception of BIM, PUMA, and BAK (Rooswinkel *et al.*, 2014). However, deletion of either BIM, PUMA, BID, or NOXA failed to prevent ABT-737-mediated cell death following *Legionella* infection, suggesting they play only a minor role, or are functionally redundant. BIK and BAD are also potential candidates, as they too are common binding partners for BCL-XL, although not MCL-1 (Rooswinkel *et*

al., 2014), and BIK is involved in displacing both MCL-1 and BCL-XL, to induce apoptosis in cells targeted by the *Escherichia coli* toxin MazF, which cleaves host mRNA to inhibit protein synthesis (Shimazu *et al.*, 2007).

How apoptosis is induced following translation inhibition varies between cell types, most likely due to the relative availabilities of different BCL-2 proteins. In many systems (Adams and Cooper, 2007; Yancey *et al.*, 2013; Okamoto *et al.*, 2014), including *Legionella* infection of macrophages, MCL-1 stability is the link between inhibition of protein synthesis and apoptosis, although its involvement is not universal (Lindqvist *et al.*, 2012). The short half-life of MCL-1 means that when protein synthesis is inhibited, cells that rely on its pro-survival activity become uniquely sensitive to the loss of other pro-survival BCL-2 proteins. Thus, in the case of *Legionella* infection, BH3-mimetics targeting BCL-XL induce rapid cell death. Combining BCL-XL-specific BH3-mimetics with novel anti-MCL-1 compounds may render BH3-mimetic therapy applicable to wide rage of infections diseases, including those where MCL-1 is not degraded.

In several microbial infections, however, BH3-mimetics that only target pro-survival BCL-XL may be a potent therapeutic option, as the inhibition of protein synthesis is one of the principal ways in which invading microbes influence host physiology [reviewed in Mohr and Sonenberg, (2012)]. It is a dual edged weapon, however, as, in addition to restricting host cell defences, it can be interpreted as a danger signal that will invoke an innate immune response (Mohr and Sonenberg, 2012). Excluding viruses, which must commandeer host translational machinery to synthesise their proteome, other pathogens that target the host protein synthesis include: *Pseudomonas aeruginosa*, via its Exotoxin A-mediated targeting of the elongation factor eEF2

(McEwan *et al.*, 2012); *Shigella*, which provokes amino acid starvation and suppression of mTORC1 to induce autophagy (Tattoli *et al.*, 2012); and *Leishmania*, via a surface glycoprotein effector GP63, which degrades mTOR (Joshi *et al.*, 2002). In addition, the fungal pathogen, *Candida albicans*, has also been shown to target the host elongation factors eIF3 and eEF2 (Martinez-Solano *et al.*, 2006).

There is also a number of pathogens that utilise pro-survival BCL-2 proteins directly to help establish infection. For example, many viruses express proteins that mimic mammalian BCL-2 proteins in order to neutralise pro-death BH3-only proteins and evade host cell apoptosis (Kvansakul and Hinds, 2013). In bacterial infections, the MAPK-mediated up-regulation and PI3K-dependent stabilisation of MCL-1 is required to prevent apoptosis of cells infected with *Chlamydia trachomatis* (Rajalingam *et al*., 2008) and, in over-expression studies, *Coxiella burnetii* has been shown to recruit BCL-2 to its vacuole membrane, where it binds to Beclin-1 and helps to prevent apoptotic cell death (Vazquez and Colombo, 2010). Thus, given the number of viral, bacterial and, possibly, fungal pathogens that utilise the pro-survival activity of BCL-2 proteins to help establish and maintain infections, it is conceivable that BH3-mimetic therapy will be applicable to a wide-range of infectious human diseases.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The over-arching aim of the work described in this thesis was to investigate the role of different programmed cell death pathways in *Legionella* infection, both in general, and during bacterial egress in particular. Multi-cellular organisms utilise programmed cell death as the first line of defence to combat invading bacteria, viruses, and parasites. Invading pathogens have developed several mechanisms to evade induction of programmed cell death pathways. This is particularly important during the early and replicative phases of infection, as the premature death of host cells interferes with bacterial replication and exposes bacteria to innate immune attack. Conversely, as resources within the cell become depleted, these same pathogens may induce host cell death to promote bacterial egress and dissemination. Thus, the nature and timing of the cell death signal employed can dramatically affect the outcome for both the host and the invading pathogen.

The main findings from my work are:

1. Flagellated *Legionella* readily induce caspase-1-dependent pyroptotic cell death, which abrogates bacterial replication.

2. *Legionella* induce an alternative flagellin/NLRC4-dependent, caspase-1/11-independent form of cell death that also restricts bacterial replication.

3. Under certain conditions, cytosolic *Legionella* activate caspase-11-dependent cell death, but this does not affect bacterial replication.

4. *Legionella* do not utilise the apoptotic or necroptotic pathways to egress from macrophages.

5. Loss of the BH3-only-targeting effector protein, SidF, does not abrogate *Legionella*-mediated killing of cells or bacterial replication.

6. *Legionella* infect, replicate, and induce cell death normally in both mice and macrophages lacking the SidF-targeting BH3-only proteins, BNIP3 and BCL-RAMBO.

7. *Legionella*-infected macrophages rely critically on the pro-survival activity of BCL-XL, but not other BCL-2 family members, for survival.

8. *Legionella* infection inhibits host protein translation, which sensitises macrophages to BCL-XL expression via depletion of MCL-1.

9. In the absence of both BCL-XL and MCL-1, *Legionella*-infected cells undergo canonical mitochondrial apoptosis, which abolishes bacterial replication and dissemination.

10. BCL-XL-targeted BH3-mimetic therapy significantly reduces *Legionella* burdens in the lungs of mice, and prevents lethal bacterial infection.

The work outlined in this thesis provides novel insights into the role of cell death signalling pathways in *Legionella* infection. However, it has also lead to the identification of a number of new areas for future research, including the following:

1. What is the flagellin/NLRC4-dependent, caspase-1/11-independent pathway that is induced by WT *L. pneumophila*?

2. What is the primary route of *Legionella* egress from spent macrophages?

3. Which BH3-only proteins interact with MCL-1 and/or BCL-XL during *Legionella* infection?

4. How are *Legionella* bacteria cleared following treatment with BH3-mimetics; is this cell-autonomous or cytokine-mediated?

5. Does BCL-XL have a similar role in human macrophages?

6. Can BH3-mimetics be used to target other intracellular pathogens, particularly those known to inhibit protein synthesis?

In this PhD project I have used live-cell imaging to follow the *Legionella*-macrophage interaction extensively. Building upon this, future work could utilise novel imaging platforms, including spinning-disc confocal and/or light sheet-microscopy to visualise these interactions in more detail. Used in combination with organellar markers, these technologies would allow elucidation of the cellular events occurring during *Legionella* egress, as well as clarification of the role of caspase-11-dependent cell death. They would also allow for testing of the hypothesis that, following treatment with BH3-mimetics, the apoptotic remnants of *Legionella*-infected macrophages are efferocytosed by bystander macrophages, which leads to the degradation of bacteria within lysosomes. *In vivo,* two-photon microscopy could be used to determine how bacteria interact with alveolar macrophages and other immune cells. To confirm that neutrophils do not mediate bacterial clearance following BH3-mimetic therapy, MPO assays or mice deficient in neutrophil recruitment could be employed, in conjunction with luciferase-labelled *Legionella* to enable visualisation of bacterial burdens. The fate of these bacteria could also be studied in more detail by infecting zebra fish containing fluorescently-labelled neutrophils and macrophages. Elucidation of these interactions will further advance our understanding of how intracellular pathogens, like *Legionella*, specifically target and regulate host cell processes to cause disease in humans, as well as aid in the identification of novel anti-bacterial therapeutic targets.

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