Programmed cell death regulation during *Salmonella* Typhimurium infections

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Abbreviations

× g	Times gravity
%	Percent
°C	Degree Celsius
AIDS	Acquired immunodeficiency syndrome
AIM2	Absent in melanoma 2
ALR	AIM2-like receptors
APAF1	Apoptotic protease activating factor 1
APC	Antigen presenting cell
ARC	Animal Resources Centre
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
BAD	BCL-2 antagonist of cell death
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2 associated X
BCL-2	B-cell leukaemia/lymphoma-2
BCL-2A1	BCL-2-related protein A1
BCL-W	BCL-2-like protein 2
BCL-XL	B-cell lymphoma-extra large
BH	BCL-2 homology
BID	BH3 interacting-domain death agonist
BIM	BCL-2 interacting mediator of cell death
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
BOK	BCL-2 related ovarian killer
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
Caspase	Cysteine-dependent aspartate-directed proteases
CD	Cluster of differentiation
CFU	Colony-forming units
cGy	Centigray

cm	Centimeter
cm^2	Square centimeter
CO_2	Carbon dioxide
Cyt c	Cytochrome c
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DED	Death effector domains
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FADD	Fas associated via death domain
FasL	Fas ligand
FCS	Foetal calf serum
FMO	Fluorescence minus one
G	Gauge
g	Gram
GAS	Gamma interferon activation site
gD	Glycoprotein D
GSDMD	Gasdermin D
GSDME	Gasdermin E
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.v.	Intravenous
IAP	Inhibitor of apoptosis protein

iBMDM	Immortalised bone marrow-derived macrophage
IFN-γ	Interferon gamma
IFNGR	Interferon gamma receptor
Ig	Immunoglobulin
IKK	Inhibitor of nuclear factor kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF1	Interferon regulatory factor 1
ISG	Interferon gamma stimulated gene
JAK	Janus kinase
kDa	Kilodalton
Km	Kanamycin
L	Liter
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M	Molar
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MARCH	Membrane associated RING-CH
max	Maximum
MCL-1	Myeloid leukemia 1
mg	Milligram
MHC	Major histocompatibility complex
mL	Milliliter
MLKL	Mixed lineage kinase domain like protein
mm	Millimeter
mM	Millimolar
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
MYD88	Myeloid differentiation primary response 88
NAIP	NLR family apoptosis inhibitory protein

ND	Not detected
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
NHMRC	National Health and Medical Research Council
NK	Natural killer
NLR	Nucleotide-binding domain and leucin rich repeat containing
NLRC4	NLR family CARD domain-containing 4
NLRP3	NLR family pyrin domain-containing 3
nm	Nanometer
NO	Nitric oxide
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
Nramp1	Natural resistance-associated macrophage protein 1
OD	Optical density
PAMP	Pathogens-associated molecular pattern
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PFA	Paraformaldehyde
pH	Potential of hydrogen
PI	Propidium iodide
PIDD	p53-induced protein with a death domain
pro-IL-1β	Pro-interleukin 1 beta
pro-IL-18	Pro-interleukin 18
PRR	Pattern recognition receptor
PUMA	p53 upregulated modulator of apoptosis
PYD	Pyrin domain
RIG-I	Retinoic acid-inducible gene-I
RIPK	Receptor-interacting serine/threonine-protein kinase
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RO	Reverse osmosis

ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s	Seconds
S. Typhimurium	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
SC	Supplementum completum
SCV	*Salmonella*-containing vacuole
SEM	Standard error of the mean
sgRNA	Single guide RNA
SMAC	Second mitochondria-derived activator of caspases
SPI	*Salmonella* pathogenicity island
spp.	Species
SseK	*Salmonella* secreted effector K
STAT	Signal transducer and activator of transcription
Str	Streptomycin
T3SS	Type III secretion system
tBID	Truncated BID
TCR	T cell receptor
Th1	T helper type 1
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
TRADD	TNF receptor 1 associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TRIF	TIR-domain-containing adaptor-inducing interferon-beta
U	Units
UoM	University of Melbourne
v/v	Volume per volume

WEHI	Walter and Eliza Hall Institute of Medical Research
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis
μg	Microgram
μL	Microliter
μm	Micrometer

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Abstract

Programmed cell death (PCD) is a highly regulated process that is vital for the development and homeostasis of multicellular organisms. It functions as an essential mechanism to remove dispensable or unfavourable cells in a coordinated manner under physiological as well as pathophysiological conditions. Importantly, this type of cellular suicide also serves as an effective defence strategy to control intracellular pathogens that aim to repurpose host cells as replicative niche and to evade extracellular immune responses. Recent findings indicate that host cells utilise multiple PCD pathways to fight invading pathogens. However, the organisation and regulation of this complex cell death network consisting of pyroptosis, apoptosis and necroptosis and their relative importance for the control and clearance of intracellular infection is not completely understood. In this thesis, we systematically investigated the cellular and molecular requirements for PCD induction and the contribution of different PCD pathways to immunity against intracellular bacteria.

We infected novel genetically modified mouse strains deficient for various combinations of PCD mediators, such as different cysteine-dependent aspartate-directed proteases (caspases), with the bacterial model organism *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) to analyse their ability to control intracellular infections. Additionally, we established *in vitro* assays to disentangle the complex interactions of the PCD network by analysing the cell death kinetics of bone-marrow derived macrophages obtained from various mouse strains in real-time and determining the intracellular bacterial burden following infection with *S.* Typhimurium. Our findings unveiled that the PCD pathways pyroptosis and apoptosis are highly interconnected and regulated with a remarkable level of redundancy. We identified that pyroptosis and apoptosis are essential for the control of *S.* Typhimurium and that molecular components of these pathways, such as caspase-1 and -8, can be used interchangeably to counteract bacterial evasion strategies. Furthermore, we investigated the underlying mechanisms that coordinate the flexible induction of pyroptosis and apoptosis during

S. Typhimurium infection. We demonstrated that caspase-2 neither plays a significant primary nor compensatory role in the regulation of cell death and control of intracellular infections. Upon excluding caspase-2 as link between PCD pathways, we evaluated means of extracellular cell death induction. The results presented in this thesis imply critical functions for cytotoxic CD4$^+$ T cells and interferon gamma (IFN-γ) in extrinsic apoptosis induction of *S.* Typhimurium infected cells.

In conclusion, our results highlight the relative importance, interconnectivity and redundancy of different PCD pathways. This study provides detailed insights into the highly complex network of PCD by unravelling new functions for several of its components and thereby defining novel mechanisms of cell death induction during infections with *S.* Typhimurium. These findings aid to discover new drug targets and develop novel treatment strategies to fight intracellular infections by enhancing essential host immune responses.

Declaration

The work presented in this thesis was conducted at the University of Melbourne in the laboratory of Prof Sammy Bedoui and at the University of Bonn in the laboratory of Prof Eicke Latz. This work was funded by the National Health and Medical Research Council, the Australian Research Council and the German Research Foundation. Sven Engel was supported by the Melbourne International Research Scholarship and the Melbourne International Fee Remission Scholarship.

This is to certify that,

(i) the thesis only comprises my original work towards the PhD, except where indicated;
(ii) due acknowledgement has been made in the text to all other material used; and
(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Melbourne, 28th April 2023 Sven Engel

Preface

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

Chapter 3: 95 %
Chapter 4: 100 %
Chapter 5: 95 %

I acknowledge the important contributions of others to the experiments presented herein:

Chapter 3: Figure 3.8 contains data obtained by Dr Paul Whitney (Bedoui laboratory, The University of Melbourne) and Dr Ranja Salvamoser (Herold laboratory, Walter and Eliza Hall Institute of Medical Research).

Chapter 5: Michele Clarke (Bedoui laboratory, The University of Melbourne) and Lindsay Kosack (Bedoui laboratory, The University of Melbourne) assisted in performing *in vivo* experiments.

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List of publications

Publications during candidature:

Functional flexibility and plasticity in immune control of systemic *Salmonella* infection.
Current Opinion in Immunology, 2023 (DOI: 10.1016/j.coi.2023.102343)
Sven Engel, Annabell Bachem, Richard A. Strugnell, Andreas Strasser, Marco J. Herold, Sammy Bedoui
This publication contains figures presented in chapter 6 of this thesis.

Caspase-2 does not play a critical role in cell death induction and bacterial clearance during *Salmonella* infection.
Cell Death and Differentiation, 2021 (DOI: 10.1038/s41418-021-00893-x)
Sven Engel, Marcel Doerflinger, Ariane R. Lee, Andreas Strasser, Marco J. Herold, Sammy Bedoui, Annabell Bachem
This publication contains data presented in chapter 4 of this thesis.

$CD4^+$ T cell immunity to *Salmonella* is transient in the circulation.
PLOS Pathogens, 2021 (DOI: 10.1371/journal.ppat.1010004)
Newton G. Peres, Nancy Wang, Paul Whitney, **Sven Engel**, Meghanashree M. Shreenivas, ian Comerford, Dianna M. Hocking, Anna B. Erazo, Irmgard Foerster, Andreas Kupz, Thomas Gebhardt, Shaun R. McColl, Stephen J. McSorley, Sammy Bedoui, Richard A. Strugnell

Flexible Usage and Interconnectivity of Diverse Cell Death Pathways Protect against Intracellular Infection.
Immunity, 2020 (DOI: 10.1016/j.immuni.2020.07.004)
Marcel Doerflinger, Yexuan Deng, Paul Whitney, Ranja Salvamoser, **Sven Engel**, Andrew J. Kueh, Lin Tai, Annabell Bachem, Elise Gressier, Niall D. Geoghegan, Stephen Wilcox, Kelly L. Rogers, Alexandra L. Garnham, Michael A. Dengler, Stefanie M. Bader, Gregor Ebert, Jaclyn S. Pearson, Dominic De

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Other publications:

MiR-21 is required for efficient kidney regeneration in fish.
BMC Developmental Biology, 2015 (DOI: 10.1186/s12861-015-0089-2)
Beate Hoppe, Stefan Pietsch, Martin Franke, **Sven Engel**, Marco Groth, Matthias Platzer, Christoph Englert

Chapter 1:

Introduction

1.1 Programmed cell death

1.1.1 Characteristics of programmed cell death

Proliferation, differentiation and the regulated elimination of cells are fundamental processes for the development, homeostasis and integrity of multicellular organisms (Ameisen, 2002, Bedoui et al., 2020). PCD plays a pivotal role in the turnover of cells and is the main disposal mechanisms for superfluous or irreversibly damaged cells. Furthermore, the removal of potentially harmful cells, such as infected or transformed cells, through the induction of organised cellular suicide is beneficial for the organism overall (Ellis et al., 1991, Strasser et al., 2000, Thompson, 1995). Aberrant PCD regulation, such as excessive cell death induction or the inability to undergo cell death, has been implied in the pathogenesis of numerous diseases, including cancer and autoimmunity as well as infectious diseases and neurodegenerative disorders (Ameisen, 2002).

Contrasting with accidental cell death, which refers to the spontaneous cellular demise by severe physical, chemical or mechanical insults, PCD is an evolutionary highly conserved and genetically regulated process involving structured signalling cascades with defined effector molecules (Ameisen, 2002, Galluzzi et al., 2018). PCD can be initiated by developmental programs and extracellular or intracellular perturbations, which stimulate membrane-bound or cytosolic sensors that induce transcriptional changes and posttranslational modifications of effector proteins (Galluzzi et al., 2018). Significant progress in the field of cell death research over the past decades has unveiled the existence of multiple forms of PCD and characterised their distinct stimuli, underlying molecular machineries, morphological characteristics and outcomes to a great extent (Bedoui et al., 2020, Galluzzi et al., 2018, Green, 2019). However, these advancements did not just unravel how surprisingly complex and multifaceted the network of PCD pathways is but also raised many new questions and uncovered unresolved aspects, which need further detailed investigation to fully understand the elaborated PCD system (Green, 2019).

The different PCD pathways can be divided into non-lytic and lytic forms of cell death (Jorgensen et al., 2017). Non-lytic cell death, such as apoptosis, ensures the coordinated disintegration and removal of dying cells, while retaining cellular integrity and preventing the induction of subsequent inflammation (Bedoui et al., 2020). In contrast to that, lytic cell death, including pyroptosis and necroptosis, leads to the breakdown of cellular integrity and the release of intracellular content, such as ribonucleic acid (RNA), deoxyribonucleic acid (DNA), proteins and lipids, into the extracellular environment. The released factors function as damage-associated molecular patterns (DAMPs) if arising from the host or as pathogen-associated molecular patterns (PAMPs) when derived from the pathogen, thereby stimulating nearby cells and triggering an inflammatory response of the immune system (Rock and Kono, 2008). The best characterised types of PCD are apoptosis, pyroptosis and necroptosis, which are regulated by caspases (McIlwain et al., 2013, Shalini et al., 2015, Van Opdenbosch and Lamkanfi, 2019). The highly conserved family of caspases consists of 12 members in humans and 10 members in mice. Caspases are proteolytic enzymes, which are synthesised as inactive precursors, called zymogens or pro-caspases, comprised of a N-terminal pro-domain and a C-terminal catalytic domain (Van Opdenbosch and Lamkanfi, 2019). The N-terminal region contains a caspase activation and recruitment domain (CARD) or death effector domains (DED) dependent on the individual caspase. The catalytic domain consists of an around 10 kilodalton (kDa) small subunit (p10) and an around 20 kDa large subunit (p20), which are connected by a linker. The generation of enzymatically inactive pro-caspases allows for their tightly regulated activation by specific stimuli and signalling pathways (Boatright and Salvesen, 2003). The activation of caspases occurs by proximity-induced dimerization, proteolytic cleavage into subunits and subsequent conformational changes (Boatright and Salvesen, 2003). Different groups of caspases have been categorised based on structural or functional similarities (McIlwain et al., 2013, Shalini et al., 2015, Van Opdenbosch and Lamkanfi, 2019). Inflammatory caspases, including caspases-1, -4, -5, -11 and -12, induce pyroptotic cell death and/or promote cytokine maturation and secretion. Caspases-4 and -5 are the human orthologues to caspase-11 found in

mice and rats. On the other hand, initiator caspases (caspases-2, -8, -9 and human caspase-10) and executioner caspases (caspases-3, -6 and -7) play an important role during apoptotic cell death. Initiator caspases induce the apoptotic signalling cascade by activating the executioner caspases, which dismantle the cell through proteolysis of numerous cellular proteins. Besides their roles in inflammation and cell death regulation, some caspases are also involved in additional cellular processes, such as survival, proliferation, differentiation or motility.

1.1.2 Mechanisms of non-lytic programmed cell death pathways

Early cell death observations were published by Vogt and Virchow in the 19th century (Virchow, 1860, Vogt, 1842). However, it was Flemming who first implied the existence of a form of cell death that is the result of intracellular chemical changes rather than external mechanical force (Flemming, 1885). Flemming was also the first to describe the process of DNA fragmentation within dying cells. Further characteristics of cell death, including nuclear fragmentation and chromatin condensation, were later reported by Glucksmann (Glucksmann, 1951). Shortly afterwards, the concept of PCD was introduced by Lockshin and Williams to characterise genetically controlled cell death processes during embryonic development (Lockshin and Williams, 1965). This first identified type of PCD was further investigated by Kerr, Wyllie and Currie and coined apoptosis (Kerr, 1965, Kerr et al., 1972). In the following years, many genes and members of the signalling cascades controlling apoptotic cell death have been identified (Diamantis et al., 2008). This led to the description of two distinct apoptotic pathways termed intrinsic and extrinsic apoptosis, which result in chromatin condensation, DNA fragmentation, phosphatidylserine exposure, membrane blebbing and the formation of apoptotic bodies (Kerr et al., 1972, Segawa et al., 2014).

Intrinsic apoptosis, often described as B-cell leukaemia/lymphoma-2 (BCL-2) regulated, mitochondrial or stress induced apoptosis, is activated in response to developmental cues, growth factor or nutrient deprivation, DNA damage and endoplasmic reticulum (ER) stress (Brumatti et al., 2010, Czabotar et al., 2014, Pihan et al., 2017, Roos et al., 2016, Singh et al., 2019). The extrinsic apoptotic pathway, also called death receptor induced apoptosis, is initiated by extracellular ligands of the tumour necrosis factor (TNF) superfamily, which includes TNF alpha (TNF-α), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Galluzzi et al., 2018).

During intrinsic as well as extrinsic apoptosis, the cellular plasma membrane integrity is maintained and dying cells are broken down into smaller apoptotic bodies in the final phase of the cell death process. The retention of intracellular content within dying cells prevents inflammatory responses. Additionally, apoptotic cells display so called “eat me” signals to phagocytes through the switch of phosphatidylserine from the inner to the outer leaflet of their plasma membrane (Green et al., 2016, Segawa and Nagata, 2015). The ligation of these signals to specific phagocyte receptors leads to the detection and rapid clearance of apoptotic cells in a process called efferocytosis (Nagata, 2018).

1.1.2.1 Intrinsic apoptosis

The intrinsic apoptotic pathway (**Figure 1.1**) is controlled by pro-apoptotic and anti-apoptotic members of the BCL-2 family (Youle and Strasser, 2008). Pro-apoptotic BCL-2 homology (BH)3-only proteins, such as BCL-2 interacting mediator of cell death (BIM), BH3 interacting-domain death agonist (BID), p53 upregulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and BCL-2 antagonist of cell death (BAD), initiate apoptosis signalling through the pro-apoptotic effectors BCL-2 associated X protein (BAX), BCL-2 homologous antagonist killer (BAK) and potentially BCL-2 related ovarian killer (BOK). In contrast to that, anti-apoptotic members, such as

BCL-2, B-cell lymphoma-extra large (BCL-XL), BCL-2-like protein 2 (BCL-W), myeloid leukemia 1 (MCL-1) and BCL-2-related protein A1 (BCL-2A1), are critical for cell survival (Galluzzi et al., 2018).

At steady state, pro-apoptotic effectors are in balance with pro-survival BCL-2 family members and therefore inactive (Adams and Cory, 2007). However, cellular stress, including DNA damage, nutrient deprivation or developmental cues, can lead to the transcriptional upregulation and/or posttranslational activation of pro-apoptotic BH3-only proteins. BIM, PUMA, NOXA and the proteolytic active form of BID, termed truncated BID (tBID), can directly activate BAX and BAK, while other BH3-only proteins, including BAD, act indirectly by binding and inhibiting the anti-apoptotic BCL-2 family members with high affinity (Galluzzi et al., 2018). This results in the liberation and subsequent oligomerisation of the apoptotic effectors BAX and BAK followed by the initiation of apoptosis (O'Neill et al., 2016). Once activated, BAX and BAK form pores into the outer mitochondrial membrane in a process called mitochondrial outer membrane permeabilization (MOMP) (Cosentino and Garcia-Saez, 2017, Czabotar et al., 2014). This causes the release of apoptogenic factors such as cytochrome c (Cyt c) and second mitochondria-derived activator of caspases (SMAC)/direct IAP binding protein with low pI (DIABLO) from the mitochondrial intermembrane space into the cytosol. Subsequently, a large protein scaffold is formed by Cyt c and apoptotic protease activating factor 1 (APAF1). This adaptor protein complex, referred to as apoptosome, enables the recruitment and activation of the initiator caspase-9. Activated caspase-9 then cleaves apoptotic effector caspases-3, -6 and -7, which subsequently proteolyse hundreds of cellular proteins leading to apoptotic cell death (Salvesen and Dixit, 1997). Following its release, SMAC/DIABLO prevents the inhibition of apoptosis by antagonising inhibitor of apoptosis proteins (IAPs), such as X-linked inhibitor of apoptosis (XIAP) (Ekert and Vaux, 2005), which are responsible for regulating the proteasomal degradation of caspases (Galban and Duckett, 2010).

1.1.2.2 Extrinsic apoptosis

The binding of extracellular members of the TNF family (TNF-α, FasL and TRAIL) to their respective receptors of the TNF receptor superfamily, TNF receptor 1 (TNFR1), Fas and TRAIL receptor (TRAIL-R), on the surface of cells can lead to the initiation of the extrinsic apoptotic pathway (**Figure 1.1**) (Ashkenazi and Dixit, 1998). These receptors harbour intracellular death domains with which they recruit adaptor proteins, such as Fas associated via death domain (FADD) or tumour necrosis factor receptor 1 associated death domain (TRADD), after ligation with their ligands. Pro-caspase-8 is then recruited to and activated within this intracellular death-inducing signalling complex called DISC (Boatright and Salvesen, 2003, Kischkel et al., 1995).

Following activation, caspase-8 cleaves downstream effector caspases-3, -6 and -7 (Strasser et al., 2000), which subsequently initiate apoptotic cell death through the cleavage of hundreds of cellular proteins in type I cells, for example thymocytes (Jost et al., 2009). However, initiation of the intrinsic pathway as effective amplification loop is required for apoptosis induction in so called type II cells, such as hepatocytes. Caspase-8 therefore cleaves the pro-apoptotic BH3-only protein BID. The proteolytic active form tBID initiates MOMP directly by activating the apoptotic effectors BAX and BAK or indirectly by binding to the anti-apoptotic BCL-2 family members (Billen et al., 2008). Subsequent activation of caspase-9 results in the increased cleavage of the effector caspases-3, -6 and -7 and cellular destruction through the proteolysis of cellular proteins (Larsen and Sorensen, 2017, Vaux and Strasser, 1996).

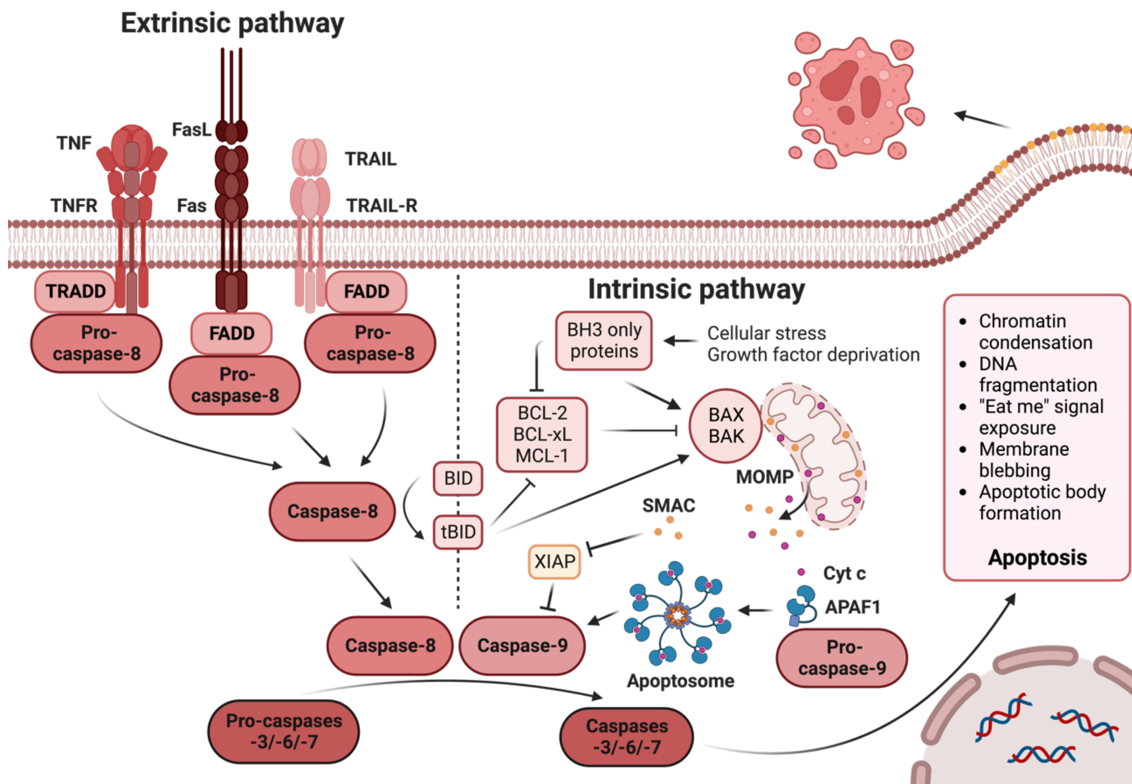


Figure 1.1: The extrinsic and intrinsic apoptosis signalling pathway.
The murine apoptotic pathway is activated by extrinsic or intrinsic signals resulting in the initiation of a caspase cascade (simplified schematic depicted). The ligation of the extrinsic tumour necrosis factor (TNF) superfamily members TNF-α, Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to their respective receptors, TNF receptor (TNFR), Fas or TRAIL receptor (TRAIL-R) leads to the recruitment of pro-caspase-8 via the adaptor proteins tumour necrosis factor receptor 1 associated death domain (TRADD) or Fas associated via death domain (FADD). The formation of this death-inducing signalling complex leads to activation of caspase-8. Active caspase-8 initiates apoptosis by cleaving the downstream effector caspases-3, -6 and -7. Besides that, caspase-8 triggers the intrinsic apoptotic pathway by cleaving the pro-apoptotic effector BH3 interacting-domain death agonist (BID) into its active form called truncated BID (tBID). Intrinsic apoptosis is initiated by deoxyribonucleic acid (DNA) damage, cellular stress stimuli, nutrient deprivation or developmental cues, which lead to the induction of pro-apoptotic BCL-2 homology 3 (BH3)-only proteins. Members of the BH3-only protein family activate BCL-2 associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK) directly or indirectly by inhibiting pro-survival BCL-2 protein family members. Unleashed BAX and BAK proteins perforate mitochondria in a process called mitochondrial outer membrane permeabilization (MOMP) thereby releasing cytochrome c (Cyt c) and second mitochondria-derived activator of caspases (SMAC). Cyt c binds to apoptotic protease activating factor 1 (APAF1) and forms together with pro-caspase-9 the apoptosome. Activated caspase 9 cleaves downstream effector caspases-3, -6 and -7. Following activation, effector caspases induce apoptotic

cell death by cleaving hundreds of intracellular proteins as well as facilitating chromatin condensation, DNA fragmentation, membrane blebbing and the exposure of “eat me” signals for subsequent phagocytosis. SMAC promotes apoptosis by inhibiting X-linked inhibitor of apoptosis (XIAP), which is responsible for the proteasomal degradation of caspases. This figure was created with BioRender.com.

1.1.3 Mechanisms of lytic programmed cell death pathways

1.1.3.1 Pyroptosis

Pyroptosis was first coined by Cookson and Brennan (Cookson and Brennan, 2001). Further investigation uncovered that pyroptotic cell death is regulated by the inflammatory caspases-1 and -11 in mice and caspases-1, -4 and -5 in humans via the canonical or non-canonical pathway (Lamkanfi and Dixit, 2014, Martinon and Tschopp, 2007, Vince and Silke, 2016, Zhao and Shao, 2016).

The canonical pyroptotic pathway (**Figure 1.2**) requires the formation of multimeric protein complexes, the inflammasomes, which subsequently facilitate the recruitment and activation of pro-caspase-1 (Lamkanfi and Dixit, 2009, Lamkanfi and Dixit, 2014, Zhao and Shao, 2016). The detection of DAMPs, PAMPs or various cellular disturbances by sensor proteins triggers inflammasome assembly (Duncan and Canna, 2018, Fernandes-Alnemri et al., 2009, He et al., 2016, Hornung and Latz, 2010). Inflammasomes are typically comprised of the involved receptors, for example nucleotide-binding domain and leucin rich repeat containing (NLRs) family members or absent in melanoma 2 (AIM2)-like receptors (ALRs), recruited pro-caspase-1 and the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) (Martinon and Tschopp, 2007, Vince and Silke, 2016). The different components bind to each other via interactions of their domains, including pyrin domains (PYD) and CARDs. Inflammasome-activated caspase-1 then cleaves the pyroptotic effector gasdermin D (GSDMD) into a N-terminal and a C-terminal fragment (Broz et al., 2020, Kayagaki et al., 2015, Shi et al., 2015). The unleashed N-terminal fragment of GSDMD translocates to the plasma membrane, where it oligomerises and forms pores (Ding et al., 2016, Man and Kanneganti, 2015). This leads to subsequent potassium efflux and water influx, loss of the membrane potential, and cellular swelling, which ultimately results in pyroptotic cell death by cell rupture and lysis (Broz et al., 2020). Morphological characteristic shared by pyroptosis and apoptosis are membrane blebbing and DNA fragmentation. However, a major difference between both pathways is that

pyroptosis also results in caspase-1 dependent cleavage of the inactive cytokine precursors pro-interleukin 1 beta (pro-IL-1β) and pro-interleukin 18 (pro-IL-18) (Galluzzi et al., 2018). The detection of extracellular DAMPs and PAMPs, such as lipopolysaccharides (LPS), by membrane-bound toll-like receptors (TLRs) leads to the activation of the nuclear factor kappa B (NF-κB) pathway via the adaptor protein myeloid differentiation primary response 88 (MYD88) (Takeuchi and Akira, 2010). The inhibitor IκB is phosphorylated by the activated inhibitor of NF-κB kinase (IKK) complex and subsequently removed via proteasomal degradation. The unleashed NF-κB complex then enters the nucleus where it induces the transcription of NF-κB-dependent target genes, such as pro-IL-1β and pro-IL-18. Following processing by caspase-1, the active forms of the cytokines leave the cell through the GSDMD induced plasma membrane pores. Pyroptosis is therefore described as an inflammatory form of PCD. The release of intracellular content during the process of cell lysis, including IL-1β and IL-18, causes an inflammatory immune response by attracting and activating various immune cells (Bergsbaken et al., 2009, Man and Kanneganti, 2015).

The non-canonical pathway of pyroptosis (**Figure 1.2**) is initiated by pro-caspase-11, which can directly bind to cytosolic LPS of Gram-negative bacteria and is activated by oligomerisation (Lamkanfi and Dixit, 2014, Shi et al., 2014, Zhao and Shao, 2016). Caspase-11 then cleaves GSDMD and thereby induces pyroptotic cell death. Notably, caspase-11, in contrast to caspase-1, is not constitutively expressed but requires transcriptional induction via interferon-α receptor or NF-κB signalling similar to pro-IL-1β and pro-IL-18 (Kobori et al., 2004, Wang et al., 1996). Besides that, caspase-11 does not convert the cytokines pro-IL-1β and pro-IL-18 into their active forms (Van Opdenbosch and Lamkanfi, 2019). However, pore formation and subsequent potassium ions influx triggers the induction of the canonical pathway, including caspase-1 activation and pro-inflammatory cytokine release.

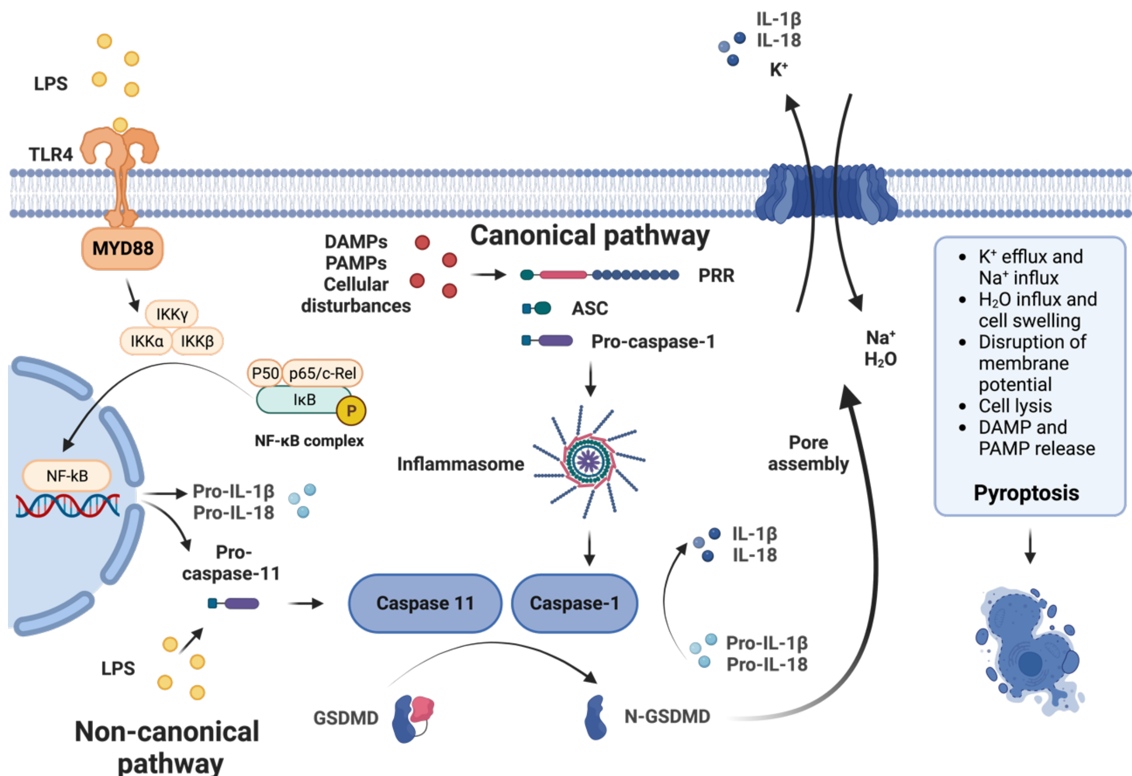


Figure 1.2: The canonical and non-canonical pyroptosis signalling pathway.

The murine canonical pyroptotic pathway (simplified schematic depicted) is induced following detection of cytosolic danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) or various cellular disturbances, for example an altered intracellular potassium (K^+) concentration, by pattern recognition receptors (PRRs), such as nucleotide-binding domain and leucine-rich repeat-containing (NLRs) receptors. Stimulated NLRs, for example NLR family CARD domain-containing 4 (NLRC4) or NLR family pyrin domain-containing 3 (NLRP3) trigger the formation of large protein platforms called inflammasomes by recruiting and binding to the adaptor protein apoptosis-associated speck-like protein containing (ASC) and pro-caspase-1. While caspase-1 is activated by inflammasomes in the canonical pathway, caspase-11 is activated via intracellular lipopolysaccharide (LPS) within the non-canonical pathway. Activated caspases-1 and -11 cleave gasdermin D (GSDMD) thereby releasing the N-terminal fragment, which subsequently translocates to the plasma membrane where it causes pore formation. The disruption of the membrane potential and water influx result in cellular lysis and pyroptotic cell death. Additionally, caspase-1 processes pro-interleukin 1 beta (pro-IL-1β) and pro-interleukin 18 (pro-IL-18) into their bioactive forms. The inflammatory cytokines are secret during pyroptotic cell death. Membrane-bound toll-like receptors (TLRs) detect extracellular DAMPs and PAMPs, such as LPS, which leads to activation of the nuclear factor kappa B (NF-κB) pathway via the adaptor protein myeloid differentiation primary response 88 (MYD88). The activated

inhibitor of NF-κB kinase (IKK) complex phosphorylates the inhibitor IκB, which results in its proteasomal degradation and the unleashing of the NF-κB complex. NF-κB enters the nucleus where it induces the transcription of NF-κB-dependent target genes, such as pro-caspase-11, pro-IL-1β and pro-IL-18. This figure was created with BioRender.com.

1.1.3.2 Necroptosis

The first proof that a regulated form of necrosis exists was provided by Vandenabeele (Vercammen et al., 1998). His work showed that murine L929 cells induced TNF-α dependent necrosis when treated with a pan-caspase inhibitor. After further characterisation, this inflammatory, lytic form of PCD was named necroptosis by Yuan and colleagues (Degterev et al., 2005).

The necroptotic cell death pathway (**Figure 1.3**) is initiated by activation of death receptors (TNFR, Fas, TRAIL-R), pathogen recognition receptors, TLRs, or receptors that recognise intracellular pathogens, for example retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). The key players in the regulation of necroptosis are receptor-interacting serine/threonine-protein kinases (RIPKs) (Newton and Manning, 2016, Vandenabeele et al., 2010, Weinlich et al., 2017). In the presence of caspase-8, necroptotic cell death is inhibited by the proteolytic cleavage of RIPK1 and RIPK3 (Ofengeim and Yuan, 2013, Vandenabeele et al., 2010). Death receptor ligation leads to the recruitment and phosphorylation of RIPK1 via the adaptor proteins TRADD and FADD. Activated RIPK1 binds to RIPK3 leading to the formation of the necrosome and subsequent autophosphorylation (Newton, 2015, Wu et al., 2014). Additionally, TLR signalling can directly activate RIPK3 through the adaptor TIR-domain-containing adaptor-inducing interferon-β (TRIF). The necrosome then phosphorylates and thereby activates the necroptotic effector mixed lineage kinase domain like protein (MLKL) (Chen et al., 2014b, Sun et al., 2012). Following conformational changes, active MLKL translocates to the plasma membrane and induces permeabilization (Cai et al., 2014, Hildebrand et al., 2014). This leads to subsequent water influx and cell swelling, which ultimately causes cell death by rupture of the plasma membrane (Chen et al., 2014b, Dondelinger et al., 2014, Su et al., 2014, Tanzer et al., 2015, Wang et al., 2014). Necroptotic cells release their intracellular content into the extracellular space, which attracts immune cells and thereby induces an inflammatory response (Newton and Manning, 2016).

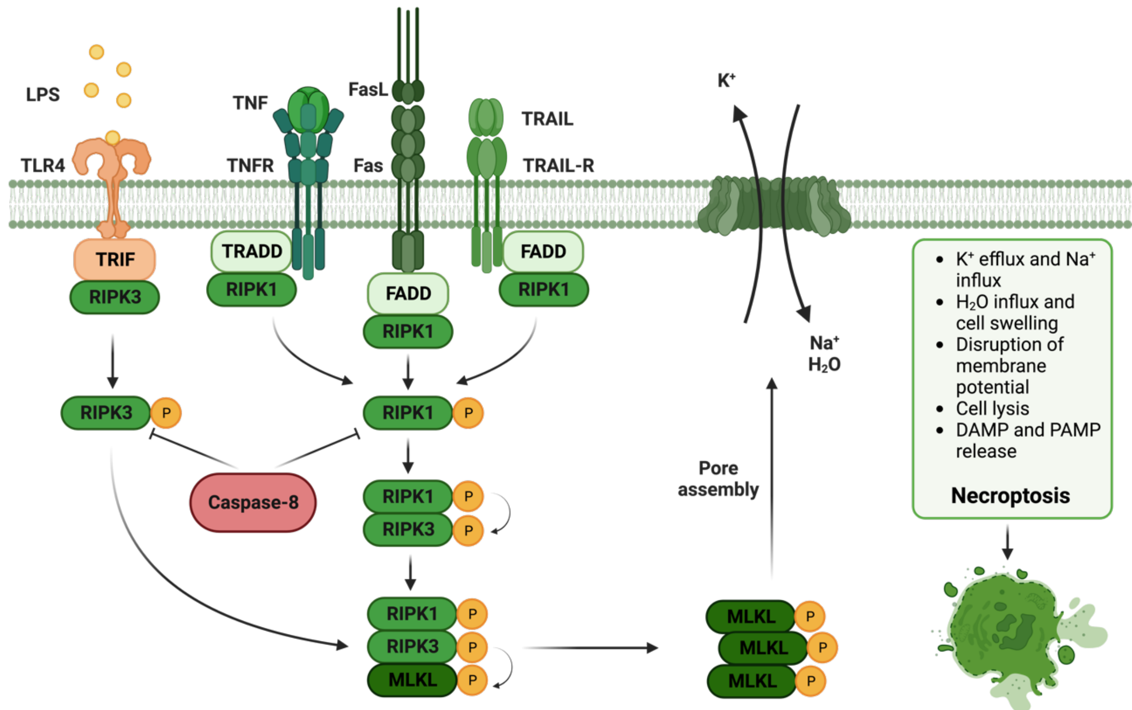


Figure 1.3: The necroptosis signalling pathway.
The ligation of the tumour necrosis factor (TNF) superfamily members TNF-α, Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to their respective receptors, TNF receptor (TNFR), Fas or TRAIL receptor (TRAIL-R), leads to the recruitment and phosphorylation of receptor-interacting serine/threonine-protein kinase (RIPK)1 via the adaptor proteins tumour necrosis factor receptor 1 associated death domain (TRADD) or Fas associated via death domain (FADD). RIPK1 then phosphorylates and activates RIPK3. Additionally, RIPK3 can be activated for example by lipopolysaccharide (LPS) stimulated toll-like receptors (TLRs) via the adaptor protein TIR-domain-containing adaptor-inducing interferon-β (TRIF). Activated RIPK3 phosphorylates the effector protein mixed lineage kinase domain like (MLKL), which translocates to and integrates into the plasma membrane. The disruption of the membrane potential and the subsequent water influx results in cellular lysis and necroptotic cell death. The induction of necroptosis (simplified schematic depicted) is inhibited in the presence of caspase-8, which is able to cleave RIPK1 and RIPK3. This figure was created with BioRender.com.

1.1.4 Important functions of programmed cell death

Initially, apoptosis, pyroptosis and necroptosis have been described as distinct PCD pathways, which are regulated by unique stimuli, comprised of specific molecular machineries and signalling cascades, therefore possessing characteristic functions and different outcomes (Green, 2019). Genetic studies implied that apoptosis is required for embryonal development and tissue homeostasis in mature organisms (Ke et al., 2018, Lindsten et al., 2000). Apoptotic cell death is also thought to be essential in preventing the development of cancer by removing mutated cells, as demonstrated by the findings that apoptosis dysregulation promotes tumour development and progression as well as resistance to therapies (Elmore, 2007, Gerl and Vaux, 2005, Neophytou et al., 2021, Pistritto et al., 2016).

Contrasting with this, pyroptosis and necroptosis are considered important mechanisms in the host defence against invading pathogens, which is at least in part thought to be due to their inflammatory nature (Brennan and Cookson, 2000, Kayagaki et al., 2015, Murphy et al., 2013, Newton and Manning, 2016, Sansonetti et al., 2000, Sun et al., 2012). The induction of lytic cellular suicide also removes the replicative niche of intracellular pathogens and exposes them to extracellular immune mediators, thereby limiting pathogen replication and dissemination as well as facilitating their detection and clearance (Thakur et al., 2019). Innate and adaptive immune responses are activated by the release of intracellular pathogens together with DAMPs and PAMPs from dying cells (Jorgensen et al., 2017, Kayagaki et al., 2015, Shi et al., 2015). The released factors enhance the capacity of macrophages and neutrophils to phagocytose and digest pathogens (Bedoui et al., 2020). Subsequent secretion of cytokines and chemokines attracts additional immune cells to the site of infection and activates them (Deets and Vance, 2021, Hachim et al., 2020).

However, recent findings challenge the concept of distinct PCD pathways with unique functions and purposes. There is growing evidence that apoptotic,

pyroptotic and necroptotic cell death processes are tightly interconnected and regulated with a remarkable level of redundancy, which allows their flexible usage in various situations (Bedoui et al., 2020, Jorgensen et al., 2017, Rauch et al., 2017, Van Opdenbosch et al., 2017).

1.1.5 Interconnectivity of programmed cell death pathways

For a long time PCD pathways have been considered to act in parallel with limited to no overlap. Recently however, the interesting concept arose that the individual PCD pathways are parts of one complex network regulating cell death in health and disease (Bedoui et al., 2020, Bertheloot et al., 2021, Samir et al., 2020). The dual role of caspase-8 as initiator of apoptosis and inhibitor of necroptosis was one of the earliest indications of interconnectivity between PCD pathways (Kaiser et al., 2011, Oberst et al., 2011, Varfolomeev et al., 1998). More recently published findings indicate that apoptotic caspases are also involved in the inflammatory response and immune defence against bacteria. There is evidence indicating that caspase-8 can be recruited to inflammasomes and induce pyroptosis by cleaving GSDMD or apoptosis in the absence of caspase-1 and GSDMD (Chung et al., 2016, Gram et al., 2019, Man et al., 2013, Man et al., 2014a, Mascarenhas et al., 2017, Pierini et al., 2012, Sarhan et al., 2018, Schneider et al., 2017). Besides that, it was shown that inflammatory caspases are able to initiate pyroptosis as well as apoptosis. Caspase-1 induces intrinsic apoptosis by proteolytically activating BID, which leads to the unleashing of BAX and BAK proteins followed by the initiation of MOMP (Heilig et al., 2020). Additionally, it was recently suggested that caspase-1 can directly cleave the apoptotic effector caspases-3 and -7 (Sagulenko et al., 2018, Taabazuing et al., 2017, Tsuchiya et al., 2019). Conversely, activated caspase-3 induces pyroptosis through cleavage of the gasdermin family member gasdermin E (GSDME) (Jiang et al., 2020, Tan et al., 2021, Wang et al., 2017). Activated GSDME forms plasma membrane pores and permeabilises the mitochondrial membrane, thereby releasing Cyt c in a positive feedback loop (Rogers et al., 2019). Furthermore,

necroptosis and extrinsic apoptosis are also highly connected. Both cell death pathways are induced through the ligation of TNF superfamily members to their respective death receptors (Galluzzi et al., 2018). While apoptosis is induced in the presence of caspase-8, necroptosis is triggered in the absence or pharmacological inhibition of caspase-8.

We require a better understanding of the complexity and redundancy of the cell death network, the existing links between different PCD pathways as well as the involvement of initiator and effector molecules therein. More studies are required to reveal so far unknown functions of apoptosis, pyroptosis and necroptosis as well as cellular and molecular requirements for their regulation in the context of intracellular infections, where the redundancy of the PCD network seems most prevalent. Further investigations will most likely identify novel pathogen and host factors involved in cell death initiation and regulation as well as reveal the role and relative importance of apoptosis, pyroptosis and necroptosis in physiological and pathological conditions. The deciphering of cellular PCD processes will uncover new drug targets and potential treatment strategies for various diseases, such as infections or cancer.

1.2 *Salmonella* species – a model organism for intracellular bacterial infection

Infectious diseases are a major threat for global health and one of the leading causes of death worldwide (Michaud, 2009). Especially infections with intracellular pathogens are a great risk factor. The intracellular lifestyle of pathogens makes treatment challenging and the constant increase in antibiotic resistances worldwide further complicates the situation (Kamaruzzaman et al., 2017, Tucker et al., 2021). Therefore, we require new strategies to fight intracellular infections by boosting the host immune system and PCD rather than targeting the pathogen itself. However, many aspects of the underlying host –

pathogen interactions and immune defence mechanisms against intracellular infections are not fully understood.

A well characterised and widely established model organism to study intracellular infections and subsequent host immune responses are *Salmonella* species (spp.) (Broz et al., 2010, Broz et al., 2012b, Franchi et al., 2006). This bacterial pathogen can be easily cultivated, genetically manipulated and flexibly used for different infection models in laboratory settings (McSorley, 2014). The genus of Gram-negative, facultative anaerobic, rod-shaped and flagellated *Salmonella* bacteria belongs to the family of *Enterobacteriaceae* and can be divided into two species, *Salmonella enterica* and *Salmonella bongori* (Dougan et al., 2011). *Salmonella enterica* can be further grouped into six distinct subspecies of which subspecies I *enterica* is responsible for the vast majority of human and animal infections (Dougan et al., 2011, Parry, 2006). Additionally, more than 1500 members of the *Salmonella enterica* subspecies *enterica* are classified as individual serovars (Fabrega and Vila, 2013).

The complete nomenclature of the *Salmonella* serovar used in this thesis is *Salmonella enterica* subspecies *enterica* serovar Typhimurium, which is commonly abbreviated as *S.* Typhimurium (Brenner et al., 2000).

1.2.1 Pathogenicity and global burden of *Salmonella* infections – gastroenteritis and typhoid fever

Salmonella spp. are typically acquired by ingestion of contaminated food and water or by contact with an infected carrier. Bacteria that survive the passage through the acidic environment of the stomach enter and colonise the lumen of the small and large intestines (Raffatellu et al., 2008). Based on the disease characteristics, infections with *Salmonella enterica* subspecies *enterica* serovars are divided into typhoidal and non-typhoidal (Gal-Mor et al., 2014).

The typhoidal *Salmonella* serovars, *Salmonella* Typhi and *Salmonella* Paratyphi, are restricted to humans and cause a systemic disease called enteric fever, also known as typhoid or paratyphoid fever respectively (Raffatellu et al., 2008). The systemic nature of the infection is due to the ability of *Salmonella* to adhere to and penetrate cells of the intestinal epithelium followed by dissemination to the intestinal lymphoid follicles and draining mesenteric lymph nodes. *Salmonella* utilises phagocytic cells as intracellular replicative niche to evade the extracellular host immune response and spread systemically to major replicative sites within the host, such as the liver and spleen (Coburn et al., 2007, Mittrucker and Kaufmann, 2000). The characteristic symptoms of enteric fever include prolonged high fever, fatigue and severe headaches, skin rash, abdominal pain as well as diarrhoea or constipation (Fabrega and Vila, 2013). Besides that, systemic *Salmonella* infections can also lead to hepatomegaly and splenomegaly (Parry, 2006). The treatment of typhoid patients, mostly young children and adolescents, includes antibiotics to inhibit bacterial growth and rehydration therapy.

In contrast to that, infections with non-typhoidal *Salmonella* serovars, such as *Salmonella* Typhimurium or *Salmonella* Enteritidis, lead to gastroenteritis in immunocompetent adults (Chen et al., 2013). These infections are typically self-limiting and restricted to the gut. Bacterial invasion of deeper tissue and systemic pathogen dissemination are largely prevented by the immune system. Common symptoms of non-typhoidal salmonellosis are fever, diarrhoea and abdominal pain. Most patients suffering from gastroenteritis recover without specific medical treatment within a week. However, non-typhoidal *Salmonella* strains can cause invasive infections and severe bacteraemia in immunocompromised individuals and young children with a high mortality rate (Feasey et al., 2012, Gordon et al., 2002, Gordon et al., 2008, Reddy et al., 2010).

The global burden of *Salmonella* infections on human health is immense, especially affecting the developing world. Yearly there are around 100 million estimated cases of non-typhoidal salmonellosis worldwide with approximately 155,000 deaths (Majowicz et al., 2010). Invasive infections with non-typhoidal

Salmonella are particularly prevalent in Africa and a major cause of morbidity and mortality in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients without access to anti-retroviral therapy (Feasey et al., 2012). Additionally, there are between 10 to 20 million estimated cases of *Salmonella* Typhi and Paratyphi infections worldwide resulting in more than 100,000 deaths every year (Browne et al., 2020, GBD 2017, 2019).

Antibiotic treatment drastically reduced the mortality of *Salmonella* infection over the past decades. However, the emergence of antibiotic resistances in typhoidal and non-typhoidal *Salmonella* strains has a great impact on the efficacy of antibiotics (Nair et al., 2018, Molbak, 2005). Especially multidrug resistant *Salmonella* strains are a serious concern (Antimicrobial-Resistance-Collaborators, 2022) and the lack of an effective vaccine against non-typhoidal serovars demands the development of additional treatment strategies, for example by targeting and improving the host immune response to intracellular infections, including PCD.

1.2.2 Mouse model for systemic *Salmonella* infection

Many aspects of today's knowledge about *Salmonella* virulence factors and the cellular and molecular immune mechanisms against *Salmonella* infections are based on experiments using an *in vivo* mouse infection model (Tsolis et al., 2011). *S.* Typhimurium infections result in a systemic typhoid fever-like disease in susceptible mice, which resembles human enteric fever in many aspects (Santos et al., 2001). Typical symptoms include hepatomegaly and splenomegaly as well as septicaemia (Jackson et al., 2010, Santos et al., 2001, Tsolis et al., 2011). Susceptible mouse strains, such as C57BL/6 and BALB/c mice, harbour a mutation within the natural resistance-associated macrophage protein 1 (Nramp1) (Vidal et al., 1996). Nramp-1 is a divalent metal ion transporter found in monocytes and macrophages, which is essential for restricting the phagolysosomal replication of intracellular pathogens (Canonne-Hergaux et al.,

1999, Hackam et al., 1998, Jabado et al., 2000). Wild-type (WT) C57BL/6 mice succumb to the infection with *S.* Typhimurium within a week due to the overwhelming bacterial replication (Dougan et al., 2011, Vidal et al., 1995). To investigate the underlying immune responses to *S.* Typhimurium infection in detail, growth attenuated strains, like SL1344 *ΔaroA* and BRD509 (*ΔaroAΔaroD* mutant of SL1344) (Strugnell et al., 1992), were generated. These *S.* Typhimurium mutants harbour defects in key metabolic enzymes, for example 3-phosphoshikimate 1-carboxyvinyltransferase encoded by the *aroA* gene, which is part of the shikimate pathway connecting glycolysis to the synthesis of aromatic amino acids (Bentley, 1990). The mutation makes *S.* Typhimurium auxotroph for aromatic amino acids and results in an overall decreased bacterial growth rate (Felgner et al., 2016). This provides enough time for the immune system of the host to counteract and induce an adequate immune response to control the invading pathogen. WT mice infected with attenuated *S.* Typhimurium strains develop a mild systemic infection and clear the bacterial pathogen within several weeks. Therefore, this model allows the detailed analysis of the innate and adaptive host immune responses to *S.* Typhimurium infections (Benoun et al., 2018, Kupz et al., 2012, Kupz et al., 2013, Kupz et al., 2014).

1.2.3 Important virulence mechanisms of *Salmonella* Typhimurium in mice

S. Typhimurium is a facultative intracellular bacterium able to survive and replicate in reticuloendothelial cells, macrophages and dendritic cells (DCs) (Mastroeni and Sheppard, 2004, Ruby et al., 2012). The bacterial pathogen possesses a wide range of virulence factors to colonise and infect host cells, disseminate systemically as well as survive within the host (Ibarra and Steele-Mortimer, 2009).

Flagella are essential for the motility of bacteria and the invasion of host cells (Schmitt et al., 2001). *S.* Typhimurium harbours two distinct flagellin genes, *fliC* and *fljB*, which encode the flagella filament structural proteins. After reaching the colon, bacteria use fimbriae to adhere to epithelial cells (Humphries et al., 2001) and subsequently penetrate the gastrointestinal mucosa by disrupting tight junctions (Jepson et al., 1995, Jepson et al., 2000). Another way to gain access to the Peyer's patches and the draining mesenteric lymph nodes is the manipulation and invasion of microfold cells (M cells) (Jensen et al., 1998, Jones et al., 1994, Penheiter et al., 1997). Important molecules for host cell invasion and intracellular survival are encoded on the *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2) (Cirillo et al., 1998, Galan, 1999, Galan and Collmer, 1999, Galan and Curtiss, 1989, Galan and Zhou, 2000, Hensel et al., 1995, Hensel et al., 1998, Mills et al., 1995, Ochman et al., 1996, Shea et al., 1996). Two needle-like type III secretion systems (T3SSs) allow *S.* Typhimurium to inject effector proteins into the cytosol of host cells to facilitate bacterial invasion by triggering membrane ruffling and actin cytoskeleton rearrangement (SPI-1 encoded T3SS) and modulate antimicrobial responses to promote intracellular survival (SPI-2 encoded T3SS).

After entering host cells, *S.* Typhimurium survives and replicates within modified phagosomes called *Salmonella*-containing vacuoles (SCVs) (Alpuche-Aranda et al., 1994, Meresse et al., 2001, Steele-Mortimer et al., 2002). Effector proteins secreted via the SPI-2 encoded T3SS inhibit further maturation of the SCV, fusion of lysosomes with the phagosome and subsequent lysosomal activities (Galan and Collmer, 1999, Gorvel and Meresse, 2001, Uchiya et al., 1999, Vazquez-Torres et al., 2000b). *S.* Typhimurium can disseminate via the bloodstream by itself or within hijacked cells (Vazquez-Torres et al., 1999), enter major replicative sites, such as liver and spleen, and thereby establish a systemic infection (Santos et al., 2001, Tsolis et al., 2011).

1.3 Host immune responses to infection with *Salmonella* Typhimurium in mice

The immune response to *S.* Typhimurium infections is complex and includes innate as well as adaptive immune cells and mechanisms, which are essential during different stages in the control and clearance of this intracellular pathogen (Dougan et al., 2011, Mastroeni, 2006). The course of infection in susceptible WT mice with growth attenuated *S.* Typhimurium strains can be separated into distinct phases irrespective of infection route or dose (Mastroeni, 2006). The induction of the innate immune response leads to the initial limitation of bacterial replication by inactivating *S.* Typhimurium through reactive oxygen species (ROS) or the complement system. However, this immediate immune response is not efficient enough to clear the infection and is followed by a phase of exponential bacterial growth. The release of various cytokines, but especially IFN-γ and TNF-α, initiates a bacterial titer plateau in major replicative sites, such as liver and spleen. The subsequent clearance of *S.* Typhimurium from the host is mediated by the adaptive immune response, especially through antigen-specific CD4$^+$ T cells, which secrete large amounts of IFN-γ thereby activating infected host cells (Dougan et al., 2011).

However, the relative importance of different PCD pathways for the regulation of innate and adaptive immune responses as well as the control and clearance of intracellular infections is not completely understood. Furthermore, while it is well established that CD4$^+$ T cells and IFN-γ are essential for the control of *S.* Typhimurium, their potential role in regulating PCD needs thorough investigation.

1.3.1 Innate immune responses against growth attenuated *Salmonella* Typhimurium

The innate immune system aids as the first line of defence against invading pathogens (Broz et al., 2012a, Pham and McSorley, 2015, Thakur et al., 2019). Innate immune responses include non-specific physical as well as chemical and cellular defence mechanisms, which are induced immediately after pathogen detection and are effective against a wide range of infections. Furthermore, innate immune processes are essential for the activation of the adaptive immune system. The family of innate immune cells includes phagocytes, mast cells, granulocytes, natural killer (NK) cells and innate lymphoid cells.

Phagocytic cells, namely monocytes, macrophages, DCs and neutrophils, are important cells of the innate immune defence against invading pathogens (Lim et al., 2017). They are responsible for the detection, ingestion and killing of bacteria. To recognise pathogens via characteristic molecular features, phagocytes are equipped with various pattern recognition receptors (PRRs) (Takeuchi and Akira, 2010). Members of the family of membrane-bound toll-like receptors (TLRs) detect distinct extracellular or endosomal bacterial PAMPs, for example peptidoglycans are recognised by TLR2 (Schwandner et al., 1999, Takeuchi et al., 1999, Yoshimura et al., 1999), LPS by TLR4 (Medzhitov et al., 1997, Park et al., 2009, Shimazu et al., 1999), flagellin by TLR5 (Hayashi et al., 2001) and bacterial DNA signatures by TLR9 (Bauer et al., 2001, Hemmi et al., 2000). The intracellular adaptor molecules MYD88 and TRIF mediate the downstream signalling of TLRs (Mogensen, 2009). Pathogen recognition by TLRs leads to enhanced activation of macrophages and increased secretion of inflammatory chemokines and cytokines, such as IFN-γ, TNF-α, IL-1, IL-6, IL-12 and IL-18. The released chemokines and cytokines coordinate the recruitment and maturation of additional immune cells from the circulation, such as DCs, monocytes, neutrophils and natural killer cells, as well as aid in the initiation of subsequent adaptive immune responses (Dougan et al., 2011, Tam et al., 2008). Besides that, cytosolic PRRs, such as RLRs and NLRs, which recognise intracellular

PAMPs and DAMPs, are of central importance for innate immune responses and pyroptosis induction in macrophages (Miao et al., 2010b, Zhao et al., 2011). The pyroptotic cell death of infected macrophages releases intracellular bacteria (Miao et al., 2010a) and potentially also captures bacteria in pore-induced intracellular traps within cell corpses (Jorgensen et al., 2016). The exposed and trapped bacteria are subsequently engulfed, killed via ROS and digested by neutrophils (Jorgensen et al., 2016, Miao et al., 2010a), which are relatively resistant to *S*. Typhimurium induced pyroptosis (Chen et al., 2014a). Additional effector function of activated neutrophils besides phagocytosis are the release of antimicrobials in a process called degranulation and the generation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004).

Another cell type of the innate immune system important for controlling intracellular infections are NK cells (Ashkar et al., 2009, Harrington et al., 2007, Schafer and Eisenstein, 1992). Stimulated through antigen presentation and IL-12 secretion by activated DCs, NK cells produce IFN-γ, which is essential for the control of *S.* Typhimurium infections. The release of IFN-γ restricts bacterial growth and is required to establish the plateau phase of *S.* Typhimurium infections with stagnating bacterial titers in different organs. Previous studies showed that this early control of *S.* Typhimurium replication can occur in the absence of T cells and B cells (Hormaeche et al., 1990, Kupz et al., 2014, Maskell et al., 1987), indicating that NK cells likely serve as important alternative source of IFN-γ during the initial stage of infection (Kupz et al., 2014, Kupz et al., 2013).

The cytokine IFN-γ, which is classified as type II interferon, is a signalling protein released for communication between host cells and a key regulator of innate and adaptive immune responses against infections (Ivashkiv, 2018, Schroder et al., 2004). The importance of IFN-γ in orchestrating the host defence against invading pathogens is highlighted by the large amount of innate and adaptive immune cells the cytokine is secreted by and the multitude of cellular effects it regulates. During the initial innate immune response to *S.* Typhimurium infections, IFN-γ is mainly produced by NK cells and antigen presenting cells (APCs) and acts as autocrine

and paracrine signal. Following secretion, IFN-γ binds to IFN-γ receptors (IFNGRs) on the surface of cells and activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. After ligation, the signalling cascade includes conformational changes of the IFNGRs that lead to recruitment and activation of JAK proteins, which subsequently phosphorylate the transcription factor STAT1. Activated STAT1 proteins form homodimers and translocate into the cell nucleus and induce target gene transcription by binding to gamma interferon activation site (GAS) elements in the promotor region of IFN-γ stimulated genes (ISGs). The upregulated genes encode proteins that promote inflammatory immune responses against pathogens and are involved in cell proliferation, differentiation and activation. Many of these IFN-γ induced factors execute essential functions in the innate and adaptive host defence against *S.* Typhimurium infections (Ingram et al., 2017). IFN-γ activates phagocytic cells as well as NK cells and promotes their antimicrobial activities. For example, IFN-γ increases phagocytosis and cytokine secretion, enhances antigen presentation via the upregulation of major histocompatibility complex (MHC) molecule expression and improves the antimicrobial killing capacity of macrophages by induction of NAPDH oxidase and inducible nitric oxide (NO) synthase (iNOS) (Flannagan et al., 2009, Reljic, 2007, Schroder et al., 2004). NAPDH and iNOS produce large amounts of ROS and NO, which inhibit and kill pathogens (Rosenberger and Finlay, 2002, Vazquez-Torres et al., 2000a).

The activation of the innate immune system, secretion of IFN-γ and formation of organised granulomas facilitates the initial control of attenuated *S.* Typhimurium strains by restricting bacterial replication (Dougan et al., 2011, Hormaeche et al., 1990, Kupz et al., 2014, Maskell et al., 1987). However, clearance of the infection requires additional mechanisms orchestrated by the adaptive immune system (Hess et al., 1996, Kupz et al., 2014).

1.3.2 Adaptive immune responses against growth attenuated *Salmonella* Typhimurium

The adaptive T cell response is primed by DCs, which are APCs of the innate immune system (Heath and Carbone, 2009). DCs that encounter pathogens or infected cells engulf and process bacterial antigen (Swart and Hensel, 2012). Activated DCs upregulate the expression of MHC as well as co-stimulatory molecules, such as CD80 and CD86, and migrate to the draining lymph nodes. The processed *S.* Typhimurium peptides are presented in the lymph nodes by DCs on MHC-I and MHC-II molecules to naïve $CD8^+$ and $CD4^+$ T cells respectively (Reis e Sousa, 2006). Following priming by DCs, T cells begin to undergo clonal expansion and differentiate into various effector lineages. Besides that, DCs can directly influence T cell effector functions, for example IFN-γ production, and migration through the secretion of IL-12 (Maldonado-Lopez et al., 1999, Shortman and Liu, 2002). Previous studies investigated the roles and importance of different adaptive immune cell subsets during *S.* Typhimurium infections. The findings indicate that antigen-specific $CD4^+$ T cells and MHC-II are required for eliminating growth attenuated *S.* Typhimurium infections, while $CD8^+$ T cells play only a minor role during late stages of infection (Hess et al., 1996, Kupz et al., 2014). Furthermore, $\gamma\delta$ T cells do not seem to be important for controlling *Salmonella* infection overall (Dougan et al., 2011, Hess et al., 1996).

T helper type 1 (Th1) cells, which are an effector $CD4^+$ T cell subset, are essential for the host defence against primary and secondary *S.* Typhimurium infections (Pham and McSorley, 2015). During pathogen clearance, Th1 cells produce cytokines, such as IFN-γ and TNF-α, required as paracrine signals to activate infected cells and promote their antimicrobial potential (McSorley, 2014, Tubo and Jenkins, 2014). Besides that, IFN-γ induces the differentiation of $CD4^+$ T cells into Th1 cells in a positive feedback loop while supressing the formation of Th2 cells, which are required for the control of extracellular pathogens (Castro et al., 2018).

Previous findings indicate that $CD8^+$ T cells play a minor protective role during primary infection with attenuated *S.* Typhimurium bacteria (Hess et al., 1996, Kupz et al., 2014, Lee et al., 2012). This cell subset can secrete IFN-γ as well as perforin and granzymes (Stenger and Modlin, 1998). Cytotoxic $CD8^+$ T cells can induce cell death in infected target cells by a process called granule-mediated lysis, typically involving the secretion of perforin, which is a pore-forming molecule capable of permeabilising the target cell facilitating the delivery of granzymes. The second killing mechanism of cytotoxic $CD8^+$ T cells is the induction of target cell apoptosis via death ligand secretion, such as TNF-α, FasL and TRAIL. After ligands bind to their corresponding death receptors on the surface of infected cells, caspase-8 is activated followed by induction of the apoptotic effector caspase cascade.

While $CD8^+$ T cells contribute to the control of primary *S.* Typhimurium infections, studies showed that they are dispensable during secondary infection (Lee et al., 2012). This is in contrast to B cells, which are not required for the clearance of primary infections with *S.* Typhimurium but are essential for protection against secondary infection (Dougan et al., 2011, Mastroeni et al., 2000, McSorley, 2014, McSorley and Jenkins, 2000, Mittrucker et al., 2000). B cells are the mediators of the adaptive humoral immune response and in charge of the production of antigen-specific immunoglobulin (Ig), also called antibodies, which are directed against pathogens (Hoffman et al., 2016). Secreted antibodies bind to invading pathogens resulting in their neutralisation by facilitating their detection by phagocytic cells or the complement system. Furthermore, studies showed that antibody-independent B cell responses are important for $CD4^+$ T cells activation (Nanton et al., 2012, Ugrinovic et al., 2003).

Even though the importance of different adaptive immune cell subsets for primary and secondary *S.* Typhimurium infection control and clearance has been described to a great extent, the underlying cellular mechanisms are not fully understood. It is necessary to understand the involvement of the adaptive immune system in the regulation of PCD during intracellular bacterial infection.

Previous findings indicate that CD4$^+$ T cells have more diverse effector functions than previously expected (Cenerenti et al., 2022, Takeuchi and Saito, 2017). Besides acting as helper cells by coordinating the activation of other immune cells and promoting their functions, the underestimated effector functions of CD4$^+$ T cells, such as target cell killing, are emerging and intensely investigated in the context of viral infections and cancer (Juno et al., 2017, Oh and Fong, 2021).

1.3.3 The role of macrophages during infection with *Salmonella* Typhimurium

Macrophages play an essential role in orchestrating innate and adaptive immune responses and are part of the first line of defence against pathogens (Gordon and Pluddemann, 2017). They are widely distributed throughout the body and display great phenotypic and functional diversity. Important macrophages functions include phagocytosis of pathogens and cellular debris, cytokine production to trigger immune responses, as well as antigen presentation and processing (Gordon, 2007, Morrissette et al., 1999, Taylor et al., 2005, Trouplin et al., 2013). The recognition of bacterial PAMPs via TLRs and the release of pro-inflammatory cytokines by various immune cells activates macrophages and induces an antimicrobial response including the production of ROS and NO, which efficiently kill intracellular bacteria (Gogoi et al., 2019). Furthermore, the cytokine driven macrophage polarisation towards a pro-inflammatory phenotype promotes the activation of the Th1 cell response of the adaptive immune system.

Macrophages are one of the most important cell types involved in *S*. Typhimurium infections, as they are the preferred target of the pathogen to establish an intracellular niche for survival and replication (Richter-Dahlfors et al., 1997, Wijburg et al., 2000). *S*. Typhimurium mutants incapable of surviving within macrophages are avirulent and fail to establish a systemic infection (Fields et al., 1986). *S.* Typhimurium uses two T3SSs for injection of bacterial effector proteins into the host cell cytosol to facilitate invasion and intracellular survival.

Macrophages sense the translocation of flagella and structural components of the SPI-1 encoded T3SS via a family of cytosolic PRRs called NLR family apoptosis inhibitory proteins (NAIPs) (Kofoed and Vance, 2011, Miao et al., 2010b, Molofsky et al., 2006, Ren et al., 2006, Sun et al., 2007, Zhao et al., 2011). Murine NAIP1 recognises the SPI-1 T3SS needle protein PrgI, NAIP2 detects the SPI-1 T3SS inner rod protein PrgJ, while NAIP5 and NAIP6 sense flagellin (Kofoed and Vance, 2011, Rauch et al., 2016, Rayamajhi et al., 2013, Yang et al., 2013, Zhao et al., 2016, Zhao et al., 2011). Following ligation, NAIPs recruit NLRC4 to oligomerise into multimeric signalling complexes called inflammasomes, which mediate caspase-1 activation, GSDMD cleavage, proteolytic maturation of the proinflammatory cytokines IL-1β and IL-18 and pyroptotic cell death (Diebolder et al., 2015, Hu et al., 2015, Zhang et al., 2015). The inflammasome adaptor protein NLRP3, which senses ROS, ion flux, lysosomal disruption and calcium signalling is another important sensor of *S.* Typhimurium infections (Broz et al., 2010, Franchi et al., 2009, Miao et al., 2010a, Zhou et al., 2011). Besides that, bacterial LPS can be directly detected by caspase-11 followed by induction of pyroptosis through the non-canonical pathway. Pyroptosis promotes the release of DAMPs and PAMPs and the induction of an inflammatory response during intracellular infection. Furthermore, cellular disintegration destroys the intracellular replicative niche and exposes intracellular bacteria, which facilitates their detection and clearance. Several studies have implied additional antimicrobial effector functions of the inflammatory caspases-1 and -11 during bacterial infections besides pyroptosis induction and cytokine secretion, which include growth inhibition of cytosolic bacteria (Thurston et al., 2016), regulation of phagosome maturation (Amer et al., 2006, Sokolovska et al., 2013), promotion of lysosomal fusion (Akhter et al., 2012, Amer et al., 2006) and reduction of cellular stiffness (Man et al., 2014a).

The underlying mechanisms of apoptotic and necroptotic cell death induction following *S.* Typhimurium infection are less well understood and published findings are often controversial. Infected macrophages can undergo delayed apoptosis in response to infection with *S.* Typhimurium. One potential SPI-2

effector that was suggested to depolymerise the actin cytoskeleton in macrophages leading to induction of apoptosis is SpvB (Browne et al., 2002, Kurita et al., 2003). Additionally, TNF-α released following *S.* Typhimurium infection can trigger the extrinsic apoptotic pathway (Wemyss and Pearson, 2019). Furthermore, it was reported that *S.* Typhimurium induces necroptosis. Following infection, type I interferon signalling is initiated, which activates RIPK1 and RIPK3 leading to necroptosis of infected macrophages (Robinson et al., 2012).

1.3.4 Programmed cell death interfering immune evasion strategies of *Salmonella* Typhimurium

Many intracellular pathogens co-evolved with our immune system and therefore developed a multitude of evasion strategies to prevent detection and inhibit host defence mechanisms (Galan, 2002). *S.* Typhimurium possesses various virulence factors and effector molecules, which intervene with a wide range of immune cells and their effector functions during different stages of the infection. This allows *S.* Typhimurium to alter host cell cytoskeleton, activation, metabolism and death in its favour to establish a systemic infection and survive within the host (Rosenberger and Finlay, 2003).

S. Typhimurium actively promotes macrophage polarisation into an anti-inflammatory phenotype, which facilitates the survival within the host (Li, 2022). Following internalisation, *S.* Typhimurium rapidly remodels its surface and downregulates SPI-1 effector proteins, such as flagella and components of the T3SS (PrgI and PrgJ), to avoid detection by intracellular sensors and subsequent inflammasome assembly and pyroptosis induction (Cummings et al., 2006, Lai et al., 2013, Miao et al., 2010a, Sporing et al., 2018). The inhibition of this fast-acting cell death pathway provides *S.* Typhimurium time to reprogram infected macrophages via SPI-2 induced mechanisms and establish an intracellular replicative niche within SCVs. By avoiding pyroptosis, *S.* Typhimurium also

prevents inflammation and the induction of additional immune responses. Furthermore, the *S.* Typhimurium effector SopB, a phosphoinositide phosphatase, can directly downregulate the NLRC4 inflammasome, which is associated with reduced cell death induction and pro-inflammatory cytokine maturation (Garcia-Gil et al., 2018, Hu et al., 2019, Perez-Lopez et al., 2013). *S.* Typhimurium also interacts with the apoptotic cell death pathway via SopB, which regulates the recruitment of Rho and Ras family guanosine triphosphate (GTP)ases and thereby promotes pro-survival Akt signalling (Chang et al., 2003, Knodler et al., 2005, Truong et al., 2018). Another *S.* Typhimurium effector that inhibits pyroptosis and apoptosis is the mitogen-activated protein kinase (MAPK) pathways inhibiting phosphothreonine lyase SpvC (Haneda et al., 2012, Mazurkiewicz et al., 2008). Death receptor induced apoptosis and necroptosis are inhibited by *Salmonella* secreted effector K (SseK)1, SseK2 and SseK3, which possess glycosyltransferase activity (Giogha et al., 2014, Kujat Choy et al., 2004, Yang et al., 2015). The family members target and modify the TNF receptor superfamily and their adaptors FADD and TRADD, which results in the inhibition of apoptosis and necroptosis signalling (El Qaidi et al., 2017, Gunster et al., 2017, Newson et al., 2019).

Furthermore, *S.* Typhimurium inhibits APC functions, antigen processing and presentation as well as T cell activation via processes mediated by the transcriptional regulator PhoP (Bernal-Bayard and Ramos-Morales, 2018, Niedergang et al., 2000, Wick et al., 1995). Intracellular bacteria downregulate their surface proteins and thereby restrict the bioavailability of antigen within APCs (Alaniz et al., 2006). *S.* Typhimurium also interferes with antigen presentation via MHC molecules by reducing their cell surface expression (Godlee et al., 2022, Gogoi et al., 2018, Lapaque et al., 2009, Mitchell et al., 2004). It was shown that the SPI-2 transmembrane effector SteD causes E3 ubiquitin ligase membrane associated RING-CH (MARCH)8-dependent ubiquitination and depletion of surface MHC-II (Bayer-Santos et al., 2016). SteD also inhibits surface expression of the co-stimulatory molecule CD86 (Bayer-Santos et al., 2016). Besides that, induction of APC death by *S.* Typhimurium

might prevent interaction with T cells and reduce T cell activation. *S.* Typhimurium also limits effector $CD4^+$ T cell activation, proliferation and survival through T cell receptor (TCR) downregulation and nutrition depletion.

The phenomenon of chronically infected individuals and asymptomatic carriers also highlights the abilities of *S.* Typhimurium to interfere and modulate the host immune system. Antibiotic treatment of *S.* Typhimurium infections induces the formation of so-called persisters. This population of bacteria are dormant, non-replicating and, what is of significant clinical concern, also tolerant to antimicrobials. Studies showed that persisters can reprogram macrophages via secretion of effectors with their SPI-2 T3SS towards an anti-inflammatory phenotype and thereby inhibit inflammatory innate immune responses (Stapels et al., 2018). Besides that, the internalisation of *S.* Typhimurium by macrophages itself induces the formation of a nonreplicating persister population (Helaine et al., 2014). This was shown to be dependent on a set of *S.* Typhimurium toxin – antitoxin modules.

S. Typhimurium is a master manipulator of cellular processes and possesses a wide range of defence strategies that target almost every aspect of the innate and adaptive immune response. However, many effector mechanisms are not well characterised and need further investigation. Due to their importance for *S.* Typhimurium control and clearance, it is of special importance to understand how PCD pathways and $CD4^+$ T cell functions are influenced by *S.* Typhimurium effectors.

1.4 Thesis aims

The high mortality of non-typhoidal *Salmonella* infections observed in immunocompromised individuals, the worldwide emergence of multi-drug resistances, and the availability of vaccines with only limited effectiveness necessitate additional strategies that improve the host immune responses

against *Salmonella* infections. There is increasing evidence that multiple forms of PCD are involved in the immune defence against intracellular infections. Furthermore, published findings indicate that different PCD pathways are interconnected, which led to speculations about the existence of a fail-safe cell death network. However, the organisation, regulation and functions of this PCD system during intracellular infections are not completely understood.

The experiments conducted in this thesis aimed to further investigate the cellular and molecular requirements of PCD induction during infections with the intracellular pathogen *S.* Typhimurium. These advances will help to unravel the complex network of PCD and identify new drug targets and potential treatment strategies to improve the outcome of intracellular infections. The results of the following aims are presented and discussed within this thesis:

1. Investigate the relative importance of different PCD pathways (pyroptosis, apoptosis and necroptosis) in *S.* Typhimurium control and clearance *in vivo* using genetically modified mouse strains deficient for different combinations of PCD regulators.
   Establish assays using bone marrow-derived macrophages to analyse cell death kinetics and intracellular burden of *S.* Typhimurium infected cells *in vitro*.

2. Analyse the role of caspase-2 in PCD regulation during *S.* Typhimurium infection *in vivo* using genetically modified mouse strains and the influence of caspase-2 on cell death kinetics and intracellular burden of infected cells *in vitro* using assays established in chapter one.

3. Characterise mechanisms of extrinsic apoptosis induction during *S.* Typhimurium infection mediated by $CD4^+$ T cells and IFN-γ which allow host cells the flexible use of different PCD pathways to ensure infection control and clearance.

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Bacteria

All *Salmonella enterica* serovar Typhimurium strains used in this study and their characteristics are listed in Table 2.1 below.

Table 2.1: *Salmonella* Typhimurium strains and their characteristics.

***S.* Typhimurium strain**	**Resistance**	**Description**	**Source/ Reference**
SL1344	Str	Wild-type strain (*rpsL hisG*)	Originally isolated from calf (Wray and Sojka, 1978)
SL1344 *ΔinvA* (χ4370)	Str, Km	SPI-1 effector deficient	R. Curtiss III (Kupz et al., 2012)
SL1344 *ΔfliCΔfljB* (χ8602)	Str	Flagellin deficient	R. Curtiss III (Kupz et al., 2012)
SL1344 *ΔaroA*	Str	Growth attenuated	N. Wang and R. A. Strugnell
SL1344 *ΔaroAΔinvA*	Str	Growth attenuated, SPI-1 effector deficient	N. Wang and R. A. Strugnell
SL1344 *ΔaroAΔfliCΔfljB*	Str	Growth attenuated, flagellin deficient	N. Wang and R. A. Strugnell
BRD509	Str	Growth attenuated, *ΔaroAΔaroD* mutant of SL1344	R. A. Strugnell (Strugnell et al., 1992)

Abbreviations: Streptomycin (Str), Kanamycin (Km)

The mutant *S.* Typhimurium strains were verified by their ability to grow in selective media (described below in subchapters 2.2.1.3 and 2.2.1.4). The introduced mutations did not affect the bacterial growth rate in Luria-Bertani (LB) broth. WT and mutant *S.* Typhimurium strains were used for *in vitro* experiments. The growth attenuated strains were used for *in vivo* experiments. All *S.* Typhimurium strains used in this study were generated and kindly provided by Professor Richard A. Strugnell (Department of Microbiology and Immunology, The University of Melbourne, Australia) and Roy Curtiss III (Department of Veterinary Medicine, University of Florida).

2.1.2 Mice

Mice were bred and maintained at the Bioresources Facility in the Department of Microbiology and Immunology at the University of Melbourne (UoM), the Walter and Eliza Hall Institute of Medical Research (WEHI) Animal Facility and the Animal Resources Centre (ARC). Mice were housed under specific pathogen-free conditions in ventilated cages, in a 12-hour light/dark cycle, receiving sterile food and water *ad libitum*. All mouse strains used in this study and their characteristics are listed in Table 2.2 below.

Table 2.2: Mouse strains and their characteristics.

Mouse strain	Description	Source	Reference
C57BL/6 (B6)	Wild-type, congenic marker Ly5.2	WEHI/ UoM	Jackson Laboratory
Ly5.1	Wild-type, congenic marker Ly5.1	ARC	
gDT-II × Ly5.1 (gDT-II.Ly5.1)	I-A^b-restricted T cell receptor (Va3.2	UoM	(Bedoui et al., 2009)

	Ja16/Vb2 Db2.1 Jb2.1) specific for HSV-1-derived glycoprotein D peptide (gD$_{(315-327)}$, IPPNWHIPSIQDA)		
TNF$^{-/-}$	TNF-α deficient	UoM	(Korner et al., 1997)
Tnf$^{-/-}$;Trail$^{-/-}$;FasL$^{gld/gld}$ (*TGLDT$^{-/-}$*)	TNF-α, Fas ligand and TRAIL deficient	WEHI/ UoM	(Ebert et al., 2020)
Casp1$^{-/-}$;Casp11$^{-/-}$	Pyroptosis deficient	WEHI	(Kuida et al., 1995)
Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$	Pyroptosis deficient	WEHI	(Salvamoser et al., 2019)
Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$; Ripk3$^{-/-}$	Pyroptosis and necroptosis deficient	WEHI	(Doerflinger et al., 2020)
Casp8$^{-/-}$;Ripk3$^{-/-}$	Extrinsic apoptosis and necroptosis deficient	WEHI	(Oberst et al., 2011)
Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$; Casp8$^{-/-}$;Ripk3$^{-/-}$	Pyroptosis, extrinsic apoptosis and necroptosis deficient	WEHI	(Doerflinger et al., 2020)
Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$; Casp8$^{-/-}$;Ripk3$^{-/-}$;Casp2$^{-/-}$	Pyroptosis, extrinsic apoptosis and necroptosis deficient, caspase-2 deficient	WEHI	(Engel et al., 2021)
Casp2$^{-/-}$	Caspase-2 deficient	WEHI	(Engel et al., 2021)

Abbreviations: Herpes simplex virus (HSV), glycoprotein D (gD)

Casp8$^{-/-}$;*Ripk3*$^{-/-}$ and *TGLDT*$^{-/-}$ mice were kindly provided by Professor John Silke (WEHI, Melbourne, Australia). All other caspase deficient mouse strains were generated and kindly provided by Professor Andreas Strasser and Professor Marco J. Herold (WEHI, Melbourne, Australia).

2.1.3 Cell lines

C57BL/6 *Cre-J2* immortalised bone marrow-derived macrophages (iBMDMs) (De Nardo et al., 2018) were modified using CRISPR/Cas9 mediated gene deletion as previously described (Aubrey et al., 2015, Kueh and Herold, 2016). Briefly, single guide (sg)RNAs targeting the genes to be deleted were designed *in silico* and cloned into an inducible lentiviral expression vector. iBMDMs were transduced with lentivirus generated in 293T cells. After expansion, infected cells were single cell sorted into medium containing 1 g/mL doxycycline hyclate to induce sgRNA expression. The single cell clones were expanded and gene deletion confirmed by western blot analysis of the targeted protein. All cell lines used in this study and their characteristics are listed in Table 2.3 below.

Table 2.3: Cell lines and their characteristics.

Cell line	Description	Source	Reference
C57BL/6 iBMDMs	Wild-type (WT)	WEHI	(De Nardo et al., 2018)
Casp8$^{-/-}$;*Ripk3*$^{-/-}$ iBMDMs	Extrinsic apoptosis and necroptosis deficient	WEHI	(Doerflinger et al., 2020)
Gsdmd$^{-/-}$ iBMDMs	Pyroptosis deficient	WEHI	(Doerflinger et al., 2020)
L929	Mouse fibroblast cells	WEHI	

All iBMDM cell lines were generated and kindly provided by Professor Marco J. Herold (WEHI, Melbourne, Australia). L929 cells were kindly provided by Professor Marc Pellegrini (WEHI, Melbourne, Australia).

2.1.4 Peptides and recombinant proteins

Table 2.4: Peptides and recombinant proteins.

Peptide/recombinant protein	Supplier
HSV-1 derived $gD_{315-327}$ (Sequence: IPPNWHIPSIQDA)	Auspep, Australia
Recombinant human IL-2	Peprotech Inc., USA
Recombinant human multimeric Fas ligand	Sapphire Bioscience, Australia
Recombinant murine IFN-γ	Thermo Fisher Scientific, USA
Recombinant murine TNF-α	Sigma-Aldrich, USA

2.1.5 Enzymes

Table 2.5: Enzymes.

Enzyme	Supplier
Collagenase Type III	Worthington, USA
DNase I	Sigma-Aldrich, USA
Trypsin/EDTA (10 X; 0.5 % trypsin, 0.2 % EDTA)	Sigma Aldrich, USA

2.1.6 Antibodies

2.1.6.1 Antibodies for $CD4^+$ T cell enrichment

Table 2.6: Antibodies for $CD4^+$ T cell enrichment.

Antibody	Clone	Supplier
CD8	53-6.7	Recombinant antibody facility, WEHI, Australia

Erythrocyte	Ter-119	Recombinant antibody facility, WEHI, Australia
F4/80	F4/80	Recombinant antibody facility, WEHI, Australia
Gr1	RB6-8C5	Recombinant antibody facility, WEHI, Australia
I-A/E	M5/114	Recombinant antibody facility, WEHI, Australia
Mac-1	M1/70	Recombinant antibody facility, WEHI, Australia

2.1.6.2 Antibodies for cell and cytokine depletion *in vitro*

Table 2.7: Antibodies for cell and cytokine depletion *in vitro*.

Antibody	Clone	Supplier
CD4	RL172	Recombinant antibody facility, WEHI, Australia
CD8	3.168	Recombinant antibody facility, WEHI, Australia
CD178 (FasL)	MFL3	BioLegend, USA
Thy1	Jlj	Recombinant antibody facility, WEHI, Australia
TNF-α	D2H4	Cell Signaling Technology, USA
CD253 (TRAIL)	N2B2	eBioscience™, USA

2.1.6.3 Antibodies for cell depletion *in vivo*

Table 2.8: Antibodies for cell depletion *in vivo*.

Antibody	Clone	Supplier
CD4	GK1.5	Recombinant antibody facility, WEHI, Australia
Thy1	T24	Recombinant antibody facility, WEHI, Australia

2.1.6.4 Antibodies and dyes for flow cytometry

Table 2.9: Antibodies and dyes for flow cytometry.

Antibody	Conjugate	Clone	Supplier
CD11b	BUV496	M1/70	BD OptiBuild™, USA
CD11b	BV711	M1/70	BioLegend, USA
CD120a (TNFR1)	PE	55R-286	BioLegend, USA
CD16/CD32 (Fc block)	-	2.4G2	BD Pharmingen™, USA
CD19	PerCP-Cy5.5	1D3	BD Pharmingen™, USA
CD3e	PerCP-Cy5.5	145-2C11	eBioscience™, USA
CD3e	APC	17A2	eBioscience™, USA
CD3e	BV711	17A2	BioLegend, USA
CD4	APC	RM4-5	BD Pharmingen™, USA
CD4	FITC	RM4-4	BD Pharmingen™, USA
CD4	PE-Cy7	RM4-5	BD Pharmingen™, USA
CD44	AF700	IM7	eBioscience™, USA
CD45.1	FITC	A20	BD Pharmingen™, USA
CD45.2	APC-eF780	104	eBioscience™, USA

CD62L	PE-Cy7	MEL-14	eBioscience™, USA
CD62L	PE	MEL-14	BD Pharmingen™, USA
CD64	BV711	X54-5/7.1	BioLegend, USA
CD80	PerCP-eF710	16-10A1	eBioscience™, USA
CD83	PE	Michel-17	eBioscience™, USA
CD86	FITC	GL1	BD Pharmingen™, USA
CD8a	BV711	53-6.7	BioLegend, USA
CD8a	Pacific Blue	53-6.7	BD Pharmingen™, USA
CD95 (Fas)	BUV737	Jo2	BD OptiBuild™, USA
F4/80	APC	BM8	eBioscience™, USA
Ly6C	AF700	HK1.4	BioLegend, USA
Ly6G	BUV395	1A8	BD Horizon™, USA
MerTK	BV421	2B10C42	BioLegend, USA
MHC-II	APC-eF780	M5/114.15.2	eBioscience™, USA
MHC-II	AF700	M5/114.15.2	eBioscience™, USA
NK1.1	PerCP-Cy5.5	PK136	BD Pharmingen™, USA
NK1.1	PE-Cy7	PK136	BD Pharmingen™, USA
Va3.2	PE	RR3-16	BioLegend, USA

2.1.7 Commercially available kits

Table 2.10: Commercially available kits.

Kit	Supplier
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega, USA
EasySep™ Mouse CD4 Positive Selection Kit II	STEMCELL Technologies, Canada

2.1.8 Media and solutions

Table 2.11: Composition of media and solutions.

Medium/solution	Composition	Supplier
Dulbecco's Modified Eagle Medium (DMEM)	DMEM + 1 g/L D-glucose + L-glutamine + 110 mg/L sodium pyruvate	Thermo Fisher Scientific, USA
Luria-Bertani (LB) agar	10 g tryptone; 5 g yeast extract; 10 g sodium chloride; 10 g agar; 1 L RO water pH 7.0 ± 0.2	Media Preparation Unit, Department of Microbiology and Immunology, The University of Melbourne, Australia
Luria-Bertani (LB) broth	10 g tryptone; 5 g yeast extract; 10 g sodium chloride; 1 L RO water pH 7.0 ± 0.2	Media Preparation Unit, Department of Microbiology and Immunology, The University of Melbourne, Australia
M9 minimal media	6 g disodium hydrogen phosphate; 3 g potassium dihydrogen phosphate; 1 g ammonium chloride; 0.5 g sodium chloride; 3 mg calcium chloride; 0.1744 g magnesium sulphate; 2 g glucose;	Media Preparation Unit, Department of Microbiology and Immunology, The University of Melbourne, Australia

	0.5 mg thiamine hydrochloride; 1 g casamino acids; 1 L RO water pH 7.0 ± 0.2	
Phosphate buffered saline (PBS)	8 g sodium chloride; 0.2 g potassium chloride; 1.15 g disodium hydrogen phosphate; 0.2 g potassium dihydrogen phosphate; 1 L ultra pure water pH 7.4 ± 0.2	Media Preparation Unit, Department of Microbiology and Immunology, The University of Melbourne, Australia
Roswell Park Memorial Institute (RPMI) 1640	10.39 g RPMI 1640 with L-glutamine and phenol red 2 g sodium bicarbonate; 1 L ultra pure water pH 6.9 ± 0.2	Media Preparation Unit, Department of Microbiology and Immunology, The University of Melbourne, Australia
Supplementum completum (SC)	23.83 g HEPES; 2×10^6 U benzylpenicillin; 2 g streptomycin; 6 g L-glutamine; 70 µL 2-β-mercaptoethanol (14.3 M); 1 L RPMI	In-house

2.1.9 Chemicals and reagents

Table 2.12: Chemicals and reagents.

Chemical/reagent	Supplier
2-β-mercaptoethanol	Thermo Fisher Scientific, USA
Benzylpenicillin	CSL, Australia
BioMag Goat Anti-rat IgG beads	QIAGEN, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
E. coli lipopolysaccharide (LPS)	Sigma-Aldrich, USA
Etanercept (Enbrel®)	Pfizer, Australia
Ethanol	Chem-Supply, Australia
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
Foetal calf serum (FCS)	CSL, Australia
Gentamicin	Sigma-Aldrich, USA
HEPES	Sigma-Aldrich, USA
Ionomycin	Sigma-Aldrich, USA
L-glutamine	Astral Scientific, Australia
Mouse serum	Thermo Fisher Scientific, USA
Neomycin	Sigma-Aldrich, USA
Paraformaldehyde (PFA)	ProSciTech, Australia
Polymyxin B	Sigma-Aldrich, USA
Propidium iodide	Sigma-Aldrich, USA
Rabbit complement	C-Six Diagnostics, USA
Rat serum	Thermo Fisher Scientific, USA
Red blood cell lysis buffer Hybri-Max™	Sigma-Aldrich, USA
Streptomycin	Sigma-Aldrich, USA
Triton X-100	Sigma-Aldrich, USA
Trypan blue	Sigma-Aldrich, USA

UltraComp eBeads™ Plus Compensation Beads	Thermo Fisher Scientific, USA
Water for Irrigation	Baxter International, USA

2.1.10 Consumables

Table 2.13: Consumables.

Consumable	Supplier
96 well plates flat bottom (black plate, clear bottom)	Sigma-Aldrich, USA
Aerosol barrier tips (10 – 1000 µl)	Interpath Services, Australia
Axygen® Microtubes (1.7 mL)	Corning, USA
Cell strainer (30 – 70 µm)	BD Bioscience, USA
CELLSTAR® Tube (50 mL)	Sigma-Aldrich, USA
Corning® Cell culture flask (25 – 175 cm^2)	Sigma-Aldrich, USA
Corning® Disposable glass Pasteur pipet	Sigma-Aldrich, USA
Corning® Microplate sealing tape	Sigma-Aldrich, USA
Costar® Stripette (5 – 25 mL)	Corning, USA
Costar® Well plates flat bottom (6 – 96 wells)	Corning, USA
Cryo.s™ Cryovials	Interpath Services, Australia
Eclipse Pipet tips (1 – 1000 µl)	Edwards, Australia
Falcon® Cell scraper (25 cm handle; 1.8 cm blade)	In Vitro Technologies, Australia
Falcon® Polystyrene round-bottom tube with lid (5 mL)	In Vitro Technologies, Australia
Glass coverslips (22 × 22 mm)	Thermo Fisher Scientific, USA
Inoculating loop (10 µl)	Labco, UK
MicroAmp™ Optical Adhesive Film	Thermo Fisher Scientific, USA

Needle (18 – 26 gauge)	Terumo, Australia
Nylon mesh (70 µm)	Madison Filter, Australia
Petri dish (90 × 14 mm)	Techno Plas, Australia
Round bottom FACS tubes (5 mL)	BD Bioscience, USA
Sarstedt Cuvettes (10 × 4 × 45 mm)	Thermo Fisher Scientific, USA
Sarstedt Tube (10 mL)	Thermo Fisher Scientific, USA
Skin Shield Gloves	Livingstone, Australia
Spreader (L shaped)	Labco, UK
Stericup® Quick Release Millipore Express® PLUS (0.22 µm PES, 500 mL)	Sigma-Aldrich, USA
Stomacher Bag (105 × 150 mm)	Labco, UK
Surgical blades	Livingstone, Australia
Syringe (1 – 10 mL)	Terumo, Australia

2.1.11 Equipment

Table 2.14: Equipment.

Equipment	Supplier
Allegra® X-12R Centrifuge	Beckman Coulter, USA
Centrifuge 5424R	Eppendorf, Germany
CLARIOstar Plus	BMG Labtech, Germany
DynaMag™-5 magnet	Thermo Fisher Scientific, USA
EasySep™ magnet	STEMCELL Technologies, Canada
Finnpipette	Thermo Fisher Scientific, USA
Force Mini Microcentrifuge	Fisher Biotec, Australia
Forceps	Australian Entomological Supplies, Australia
Glass spreader	Sigma-Aldrich, USA
HERAcell VIOS 160i CO_2 Incubator	Thermo Fisher Scientific, USA

Inverted microscope CKX31	Olympus Lifescience, Japan
Laboratory water bath	Thermoline Scientific, Australia
Leica DMI4000 B Inverted microscope	Leica, Germany
LSRFortessa™ cell analyser	BD Bioscience, USA
Neubauer Improved Brightline haemocytometer	Thermo Fisher Scientific, USA
Safemate 1.2 Vision Class II Biological Safety Cabinet	Laftech, Australia
Scan® 100 Manual colony counter	Interscience, France
Sissors	Australian Entomological Supplies, Australia
Upright microscope CX23	Olympus Lifescience, Japan
Vortex mixer VM1	Ratek Instruments, Australia

2.2 Methods

2.2.1 Bacterial culture

2.2.1.1 Bacterial growth conditions

S. Typhimurium strains were grown shaking at 180 revolutions per minute (rpm) in LB broth or static on LB agar plates at 37 °C. The culture media were supplemented with 50 µg/mL streptomycin. *S.* Typhimurium strains used in this study were resistant against streptomycin.

2.2.1.2 Preparation of bacterial glycerol stocks

To prepare glycerol stocks of *S.* Typhimurium cultures for long-term storage, each strain was streaked out onto a LB agar plate containing 50 µg/mL

streptomycin and incubated at 37 °C overnight. Single colonies were picked and transferred into 10 mL LB broth containing 50 µg/mL streptomycin. Bacterial cultures were incubated shaking at 37 °C overnight and 80 % (v/v) sterile glycerol was added to the bacterial cultures in a 1:1 ratio directly before freezing. Aliquots of these glycerol stocks were stored at -80 °C. The viability of bacteria was determined by streaking out samples of the frozen aliquots onto LB agar plates containing 50 µg/mL streptomycin followed by incubation at 37 °C overnight.

To prepare aliquots for *in vivo* infections, overnight cultures of the *S.* Typhimurium glycerol stocks were prepared by inoculating 10 mL LB broth containing 50 µg/mL streptomycin. 100 µL of the overnight cultures were then added to 10 mL of fresh LB broth with 50 µg/mL streptomycin and incubated shaking at 37 °C for 3 – 4 hours. The optical density of the culture at 600 nm (OD_{600}) was determined and 80 % (v/v) sterile glycerol was added in a 1:10 ratio to the bacteria during the exponential growth phase. Aliquots were frozen and kept at -80 °C for long-term storage. The concentration of viable bacteria per aliquot was determined by streaking out serial dilutions onto LB agar plates.

2.2.1.3 Bacterial motility assay

Bacteria were stab-inoculated into semi-solid LB agar (0.2 % agar) supplemented with appropriate antibiotics and incubated at 37 °C overnight to determine the motility of *S.* Typhimurium strains. Flagellin-deficient, non-motile bacteria remained confined to the inoculation side. In contrast to that, flagellated, motile bacteria were able to colonise the entire culture medium.

2.2.1.4 Bacterial growth in selective media

Bacteria were grown at 37 °C in M9 minimal media (supplemented with 1.3 mM L-histidine) with or without the addition of an aromatic amino acid mix (0.25 mM L-phenylalanine, 0.25 mM L-tyrosine, 0.1 mM L-tryptophan, 0.073 mM p-aminobenzoic acid and 0.065 mM p-hydroxybenzoic acid) to determine if *S.* Typhimurium strains are auxotroph for aromatic amino acids. *S.* Typhimurium strains with mutations in the *aroA* and *aroD* genes were unable to grow in media without the aromatic amino acid mix.

2.2.2 Animal experiments

All animal experiments were approved by The University of Melbourne Animal Ethics Committee (Animal ethics numbers 1714194 and 23650) and complied with the Prevention of Cruelty to Animal Act (1986) and the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). Age- and sex-matched animals between 6 and 14 weeks of age at the beginning of experiments were used for *in vivo* and *in vitro* studies. Imported mice from other animal facilities were kept for at least one week to adapt to the new environment before starting the experiments. Mice were euthanised by CO_2 asphyxiation at the experimental end point or based on disease severity (including weight loss of more than 15 %, body condition, physical appearance and behaviour), which is described as 'mouse survival'.

2.2.2.1 Intravenous injection

For intravenous injections, mice were warmed up using a heat lamp (32 °C) until tail vein was dilated and then placed into a restrainer. The injection side was

cleaned with ethanol and the inoculum was slowly injected into the tail vein in a volume of 200 µL sterile PBS using a 26 gauge (G) needle.

2.2.2.2 Intraperitoneal injection

For intraperitoneal injections, mice were scruffed and the injection site was cleaned with ethanol. Substances were injected using a 26 G needle in a volume up to 200 µL.

2.2.2.3 Blood collection

For the collection of blood, mice were scruffed and the submandibular vein was punctured using a 26 G needle or cardiac puncture was performed immediately after euthanasia. The blood samples were passed through heparinised capillaries to prevent blood clotting for downstream analysis.

2.2.2.4 Generation of bone marrow chimeric mice

Recipient Ly5.1 mice were lethally irradiated with 2 doses of 550 cGy 3 hours apart (WEHI irradiation facility) and reconstituted with intravenously injected 2.5 – 5 × 10^6 T cell-depleted bone marrow cells from donor mice expressing the congenic marker Ly5.2. The donor bone marrow was incubated with anti-CD4 (clone RL172), anti-CD8 (clone 3.168) and anti-Thy1 (clone Jlj) antibodies on ice for 30 minutes to deplete T cells and prevent graft-versus-host disease. Cells were pelleted and resuspended in 1 mL rabbit complement at 37 °C for 20 minutes to lyse T cells. After incubation, cells were washed and resuspended in sterile PBS before injection. On the following day, mice were depleted of radioresistant T cells by intraperitoneal injection of 100 µL anti-Thy1 antibody

(clone T24). Chimeric mice were maintained for 6 weeks on antibiotic water containing 25 mg/L neomycin sulphate as well as 10 mg/L polymyxin B sulphate and allowed to reconstitute for at least 8 weeks. Reconstitution of the bone marrow (> 90 %) was verified via flow cytometry before mice were infected with *S.* Typhimurium.

2.2.2.5 *In vivo* inhibition of TNF-α

TNF-α was blocked *in vivo* by intraperitoneal injections of the fusion protein etanercept. Mice were injected with 200 µg of etanercept in a volume of 200 µL sterile PBS twice weekly. The first dose of etanercept was injected 24 hours before infection to ensure sufficient TNF-α depletion at the begin of the experiment. Etanercept was kindly provided by Professor Marc Pellegrini (WEHI, Melbourne, Australia).

2.2.2.6 *In vivo* depletion of $CD4^+$ T cells

The $CD4^+$ T cell subsets was depleted from mice by intraperitoneal injections of 100 µg of anti-CD4 monoclonal antibodies (clone GK1.5) in sterile PBS twice weekly. To ensure sufficient depletion at time of infection, antibodies were injected 72 and 48 hours before infection. The effectiveness of depletion was routinely confirmed by flow cytometry.

2.2.2.7 Infection of mice

Although *Salmonella* spp. are typically acquired via the faecal-oral route, the outcome of the infection with attenuated strains is independent of the infection route (Hess et al., 1996, Kupz et al., 2014, Mastroeni, 2006, Weintraub et al.,

1997). We used an intravenous infection model to ensure high reproducibility between experiments and to analyse the immune response involved in pathogen clearance from systemic organs.
Directly before infection, previously prepared bacterial glycerol aliquots for *in vivo* infections were thawed, pelleted at 10,000 × g for 5 minutes at 4 °C and washed three times with 1 mL sterile PBS. The aliquots were diluted according to the prior determined total number of viable bacteria and 200 colony forming units (CFU) of *S.* Typhimurium were injected into the tail vein in a volume of 200 µL sterile PBS. Each inoculum was plated out onto LB agar plates supplemented with 50 µg/mL streptomycin and incubated at 37 °C for 24 hours to confirm the accuracy of the infectious dose.

2.2.2.8 Determination of bacterial titers *ex vivo*

Infected animals were euthanised with CO_2 at indicated time points post-infection to determine the number of viable *S.* Typhimurium bacteria in livers, spleens and blood of infected mice. Organs were removed aseptically and transferred into sterile homogenising bags. The weights of harvested livers and spleens were recorded and organs subsequently homogenised for 15 minutes in 3 – 5 mL of sterile ice-cold PBS using a Stomacher 80 homogeniser. The homogenates were then serially diluted and duplicates were streaked out onto LB plates containing 50 µg/mL streptomycin. To determine viable *S.* Typhimurium bacteria in the circulation of infected mice, heparinised blood was serially diluted and duplicates were streaked out onto LB plates containing 50 µg/mL streptomycin. Inoculated agar plates were incubated at 37 °C for 24 hours. The colonies formed by viable bacteria were counted and expressed as CFU/organ.

2.2.2.9 Preparation of mouse tissues for flow cytometry

Spleens were collected in ice-cold RPMI supplemented with 10 % FCS and 5 % SC and dissociated with a surgical blade and digested in 1 mL of collagenase III/DNase I (1 mg/L and 20 µg/mL) by pipetting up and down for 20 minutes at room temperature (RT) instead. 600 µl of 0.1 M EDTA (pH 7.2) was added and splenocytes further incubated by pipetting up and down for 5 minutes at RT. Cells were filtered through a 70 µm cell strainer and washed with PBS. After centrifugation, splenocytes were resuspended in 3 mL of red blood lysis buffer and incubated for 1.5 minutes at RT. Cells were washed with PBS after lysis, centrifugated for 5 minutes at 1600 rpm and 4 °C and counted after resuspension.

Blood was incubated twice in 1 mL of red blood cell lysis buffer at RT for 3 minutes, centrifugated for 5 minutes at 1600 rpm and 4 °C and washed with PBS supplemented with 10 % BSA and 5 mM EDTA (fluorescence activated cell sorting (FACS) buffer) before staining.

2.2.3 Cell culture

2.2.3.1 Cell culture conditions

Bone marrow-derived macrophages (BMDMs), iBMDMs and L929 cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM with 1 g/L D-glucose, L-glutamine and 110 mg/L sodium pyruvate) supplemented with 10 % heat inactivated FCS, 100 U/mL benzylpenicillin and 200 µg/mL streptomycin at 37 °C and 5.6 % CO_2. Cells were washed with sterile PBS, detached using trypsin/EDTA or a cell scraper and centrifugated for 5 minutes at 1600 rpm and 4 °C.

$CD4^+$ T cells were cultured in RPMI 1640 media supplemented with 10 % heat inactivated FCS (RP-10) and 5 % SC at 37 °C and 6.5 % CO_2. Cells were washed with sterile PBS and centrifugated for 5 minutes at 1600 rpm and 4 °C.

2.2.3.2 Preparation of L929-conditioned medium

The supernatant of L929 cells containing macrophage colony-stimulating factor (M-CSF) was used for the differentiation of murine bone marrow cells into macrophages. For the generation of conditioned medium, a vial of L929 cells was thawed and cultured in complete DMEM. Cells were expanded in 75 cm^2 flasks at 37 °C and 5.6% CO_2 until 80 – 90 % confluency. Cells were split 1:5 into 175 cm^2 flasks with 50 mL of complete DMEM and grown for 7 days before the supernatant was harvest, centrifugated for 10 minutes at 1600 rpm and filter sterilised. The L929-conditioned medium was aliquoted and stored at -20 °C.

2.2.3.3 *In vitro* generation of bone marrow-derived macrophages

For the *in vitro* generation of murine BMDMs, bone marrow was flushed from femurs and tibiae using a 26 G needle and filtered through a 70 µm cell strainer to obtain a single cell suspension. Cells were resuspended in complete DMEM supplemented with 15 % L929-conditioned medium (as a source of M-CSF) and cultured at 2×10^6 cells/mL for 6 to 7 days in non-tissue culture treated dishes at 37 °C and 5.6 % CO_2. The culture medium was replaced on day 3. Macrophage differentiation was validated by confirming the expression of CD11b and F4/80 via flow cytometry.

For analysis, differentiated BMDMs were seeded in 500 µL or 100 µL antibiotic-free DMEM into 24- or 96-well plates at a density of 2×10^5 or 1×10^5 cells/well,

respectively. iBMDMs were seeded into 96-well plates at a density of 5×10^4 cells/well in 100 µL of antibiotic-free DMEM. Cells were allowed to rest for at least 12 hours before infection or downstream analysis.

2.2.3.4 *In vitro* stimulation of macrophages

BMDMs and iBMDMs were stimulated *in vitro* with IFN-γ (100 ng/mL), TNF-α (50 ng/mL) and FasL (1 µg/mL) alone or in combination for indicated durations.

2.2.3.5 *In vitro* infection of macrophages

S. Typhimurium was grown shaking at 37 °C overnight in LB broth supplemented with 50 µg/mL streptomycin. For *in vitro* infections of primary BMDMs, bacteria were pelleted at 3750 rpm for 10 minutes at 37 °C, washed and resuspended in sterile, pre-warmed PBS. OD_{600} was determined using a spectrophotometer and bacteria were diluted in pre-warmed, antibiotic free, sterile DMEM to the desired multiplicity of infection (MOI). Following addition of the bacteria to the cells, culture plates were centrifugated at 1600 rpm for 5 minutes at 37 °C to synchronise the infection. Each inoculum was plated out onto LB agar plates supplemented with 50 µg/mL streptomycin and incubated at 37 °C for 24 hours to confirm the accuracy of the infectious dose.

2.2.3.6 $CD4^+$ T cell enrichment

$CD4^+$ T cells were enriched from spleens and lymph nodes of naïve gDT-II mice. Single cell suspensions of harvested organs were prepared by mechanical disruption and filtering through a 70 µm cell strainer. Splenocytes were incubated with 3 mL of red blood cell lysis buffer for 3 minutes at RT. Cells were washed

with PBS containing 2 % FCS and 2 mM EDTA and each organ resuspended in 1 mL of $CD4^+$ T cell negative enrichment antibody cocktail (anti-Mac-1 (M1/70, anti-F4/80 (F4/80), anti-erythrocytes (TER-119), anti-GR-1 (RB6-8C5), anti-I-A/E (M5/114), anti-CD8 (53-6.7)) on ice for 30 minutes. Following incubation, cells were washed and resuspended in PBS containing 2 % FCS and 2 mM EDTA. Goat anti-rat IgG-coupled magnetic beads were added to the cells in a 6:1 bead to cell ration and incubated rotating at 4 °C for 15 minutes. Tubes were loaded onto the DynaMag™-5 magnet and allowed to rest for 3 minutes before the supernatant containing enriched $CD4^+$ T cells was collected. Cells were counted and resuspended at a density of 1×10^8 cells/mL for positive enrichment following the EasySep™ Mouse CD4 Positive Selection Kit II protocol. In brief, 50 µL of rat serum was added to each sample. Cells were then mixed with 50 µL of monoclonal antibody mix per 1 mL of sample and incubated at RT for 5 minutes. RapidSpheres™ particles were added at a concentration of 30 µL/mL of sample. Samples were mixed and incubated at RT for 3 minutes. Magnetic particle labelled $CD4^+$ T cells were enriched by placing the samples onto the EasySep™ magnet at RT for 3 minutes and washing cells three times with PBS containing 2 % FCS and 2 mM EDTA. Enriched $CD4^+$ T cells were resuspended in RP-10. Purity (> 95 %) of the $CD4^+$ T cell culture was confirmed via flow cytometry.

2.2.3.7 *In vitro* activation of $CD4^+$ T cells

Naïve C57BL/6 splenocytes were pulsed with 5 µM $gD_{315\text{-}327}$ peptide for 45 minutes at 37 °C. Enriched gDT-II cells were co-cultured with peptide-pulsed splenocytes in RP-10 and 0.15 mg/mL LPS for activation. Starting from day 2 post-activation, cells were split daily and 12.5 U/mL of IL-2 was added. Following activation for 6 – 7 days, cells were washed and resuspended in DMEM for $CD4^+$ T cell – macrophage co-culture. Activation of the gDT-II cell culture was confirmed via flow cytometry.

2.2.3.8 *In vitro* co-culture of bone marrow-derived macrophages and $CD4^+$ T cells

Prior to co-culture with activated TCR transgenic $CD4^+$ T cells, differentiated BMDMs were pulsed with 5 μM $gD_{315-327}$ peptide for 45 minutes at 37 °C. BMDMs and gDT-II cells were washed and resuspended in fresh media. gDT-II cells were added in indicated effector cell to target cell ratios to BMDMs.

In some experiments, blocking antibodies against TNF-α (5 μg/mL), FasL (2 μg/mL) and TRAIL (10 μg/mL) were added alone or in combination to gDT-II cells prior to the co-culture with BMDMs.

2.2.4 Functional *in vitro* assays

2.2.4.1 Determination of intracellular bacterial counts *in vitro*

BMDMs were infected with the indicated *S.* Typhimurium strain and MOI in antibiotic-free medium. After incubation at 37 °C for 1 hour, cells were washed twice with PBS and the medium was replaced for 1 hour with DMEM (+ 10 % FCS) containing 100 μg/mL gentamicin, followed by DMEM (+ 10 % FCS) with 10 μg/mL gentamicin for the remaining time to prevent growth of extracellular bacteria. At indicated time points uninfected and *S.* Typhimurium infected BMDMs were washed twice with PBS and lysed in 1 mL of 1 % TritonX-100 in distilled water for 15 minutes. The numbers of replicating intracellular bacteria were determined by serially diluting and plating the cell lysates onto LB agar plates supplemented with the appropriate antibiotics. The plates were incubated at 37 °C for 24 hours. Viable bacteria were counted and expressed as CFU/well.

2.2.4.2 Lactate dehydrogenase release assay

BMDMs were infected with the indicated *S.* Typhimurium strain and MOI in antibiotic-free culture medium and 50 µg/mL gentamicin was added 1 hour after infection to remove extracellular bacteria. The lactate dehydrogenase (LDH) release of BMDMs was determined at the indicated timepoints using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. The percentage of cytotoxicity at each timepoint was calculated by normalising the LDH release of *S.* Typhimurium-infected cells to the maximum LDH release control.

2.2.4.3 Real-time cell death kinetics assay measured by propidium iodide incorporation

BMDMs and iBMDMs were seeded into black 96-well plates with clear bottom and infected with the indicated *S.* Typhimurium strain and MOI in antibiotic-free medium containing propidium iodide (PI; 6 µg/mL per well). Gentamycin was added 1 hour after infection to inhibit extracellular bacterial growth. Cell death kinetics of uninfected (negative control) and *S.* Typhimurium infected cells were measured by the incorporation of PI using the CLARIOstar Plus microplate reader. For some experiments, uninfected and infected BMDMs were peptide pulsed and co-cultured with TCR transgenic $CD4^+$ T cells at indicated effector cell to target cell ratios to measure $CD4^+$ T cell mediated macrophage cell death kinetics. Cell death kinetics of non-pulsed (negative control) and peptide-pulsed cells were measured by the incorporation of PI using the CLARIOstar Plus microplate reader.

The percentage of cell death at each time point was calculated by normalising the PI uptake of infected/non-pulsed cells to that of 4 % paraformaldehyde treated cells used as a control for maximum cell lysis. Samples were measured in

triplicates. The focal height was adjusted using the positive control as reference and the measurement was performed with the following protocol settings:

Table 2.15: Protocol settings for real-time *in vitro* killing assay.

Measurement type	Fluorescence
Microplate name	COSTAR 96
Read mode	Bottom optic
Scan mode	Spiral scan
Scan diameter (mm)	5
Number of cycles	250
Cycle time (s)	300
Number of flashes per well and cycle	50
Excitation (nm)	535 +/- 15
Emission (nm)	617 +/- 20
Dichroic filter (nm)	574.8
Temperature (°C)	37
CO_2 (%)	5.6

2.2.5 Flow cytometry

2.2.5.1 Cell surface marker staining

Single cell suspensions were stained in 50 – 100 µL of FACS buffer containing a mix of fluorochrome-conjugated antibodies and Fc-block to prevent unspecific binding. Following incubation at 4 °C for 30 minutes, cells were filtered and washed with FACS buffer and resuspended in FACS buffer containing 1 µg/mL PI to exclude non-viable cells. Single colour-stained compensation controls were prepared using cells or compensation beads.

2.2.5.2 Measurement and analysis of cell populations

All compensation controls and samples were measured using the BD LSRFortessa™ Cell Analyser. Data was analysed with the software FlowJo™ 10.

2.2.6 Quantification and statistical analysis

GraphPad Prism v9.0.1 was used to generate figures and perform statistical analysis. Bacterial colony forming units were log transformed before analysis. Data are shown as mean + standard error of the mean (SEM). Please refer to the figure legends for description of the statistical test used, sample size (n) and calculated statistical significance (p values). $p \leq 0.05$ was considered to indicate statistical significance: not significant (ns), $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Chapter 3:

The relative importance of pyroptosis, necroptosis and apoptosis for *Salmonella* Typhimurium control and clearance

3.1 Background

The induction of PCD is an important host immune defence mechanism to control intracellular infections, such as *S*. Typhimurium. Following host cell invasion or engulfment by phagocytic cells, bacteria modify host cell phagosomes by secretion of effector molecules to establish an intracellular replicative niche, called SCV (Haraga et al., 2008, Kumar and Valdivia, 2009, Malik-Kale et al., 2011, Richter-Dahlfors et al., 1997, Salcedo et al., 2001, Steele-Mortimer, 2008). Intracellular survival and replication within phagocytes, in particular macrophages, is essential for the pathogenesis and virulence of *Salmonella* (Fields et al., 1986, Haraga et al., 2008, Leung and Finlay, 1991). However, infected cells can undergo different forms of PCD, including pyroptosis, necroptosis and apoptosis, to remove this replicative niche (Bergsbaken et al., 2009, Jorgensen and Miao, 2015, Jorgensen et al., 2017, Lamkanfi and Dixit, 2010). This limits bacterial replication, exposes pathogens to extracellular mediators of the immune system and facilitates their detection and clearance (Thakur et al., 2019). Therefore, many intracellular pathogens have developed strategies to manipulate and inhibit host cell death pathways to promote bacterial survival and proliferation (Kumar and Valdivia, 2009, Lamkanfi and Dixit, 2010, Wemyss and Pearson, 2019), including the downregulation of PAMPs, such as flagellin or components of the T3SS, that are detected by host sensors (Cirillo et al., 1998, Cummings et al., 2006, Galan, 1999, Galan and Collmer, 1999, Galan and Curtiss, 1989, Galan and Zhou, 2000, Hensel et al., 1998, Lai et al., 2013, Miao et al., 2010a, Mills et al., 1995, Ochman et al., 1996, Shea et al., 1996).

The relative importance of each PCD pathway for immunity against *S*. Typhimurium has recently been questioned and requires a thorough and systematically investigation. The role of pyroptosis and the contributions of the inflammatory caspases-1 and -11 in response to intracellular infections were previously analysed in several *in vivo* studies (Man et al., 2017). Collectively, findings showed that *Casp1*$^{-/-}$, *Casp11*$^{-/-}$ and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$ mice are more susceptible to oral infections with several Gram-negative bacteria, including

S. Typhimurium, indicated by increased bacterial burden at different sites (Broz et al., 2010, Broz et al., 2012b, Knodler et al., 2014, Lara-Tejero et al., 2006, Man et al., 2014b, Raupach et al., 2006). Taken together, these findings indicate that pyroptosis and the secretion of IL-1β and IL-18 mediated by the activation of inflammatory caspases-1 and -11 contribute to immunity against *S*. Typhimurium. Furthermore, pyroptosis was referred to as the primary mechanism for bacterial clearance (Jorgensen et al., 2017).

Nevertheless, some of these studies implied that *S*. Typhimurium infections can be controlled even under conditions where inflammasome-driven pyroptosis is absent (Broz et al., 2010, Broz et al., 2012b) indicating that whereas pyroptosis is required to control bacterial replication it is negligible for their clearance. To confirm the redundancy of pyroptosis in *S*. Typhimurium control and clearance *in vivo*, we previously infected caspase-1 and -11 deficient mice with the growth attenuated *S*. Typhimurium strain BRD509 intravenously and measured bacterial titers in liver and spleen over a period of 12 weeks post-infection. The infection of WT mice with BRD509 results in a mild systemic infection that is controlled with liver and spleen as major bacterial replication sites (Kupz et al., 2014). As expected, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$ mice showed a slightly impaired *S*. Typhimurium control compared to WT mice indicated by significantly elevated bacterial titers at the peak of infection (Doerflinger et al., 2020). However, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$ mice cleared the infection similarly to WT mice within 12 weeks (Doerflinger et al., 2020). These results imply that either PCD-independent antimicrobial host defence mechanisms are sufficient to control the infection or that additional cell death mechanisms compensate for the loss of pyroptosis, which would highlight the host’s ability to counteract evasion strategies used by bacteria to prevent immune responses (Bedoui et al., 2010). Recent additional findings implied the existence of a complex fail-safe systems that involves various caspases and different PCD pathways (Jorgensen et al., 2017, Rauch et al., 2017, Van Opdenbosch et al., 2017).

The above summarised *in vivo* findings and concluded hypotheses regarding the importance of pyroptosis and inflammatory caspases-1 and -11 during *S*. Typhimurium infection are further supported by the results of several *in vitro* studies. Initial publications reported that *S*. Typhimurium SL1344 induces inflammasome driven pyroptosis and that macrophages deficient for NLRC4, caspase-1 or GSDMD are resistant to *S*. Typhimurium induced cell death (Franchi et al., 2009, Jesenberger et al., 2000, Lara-Tejero et al., 2006, Lightfield et al., 2008, Mariathasan et al., 2004, Miao et al., 2006). Furthermore, it was shown that pyroptotic macrophage cell death is dependent on flagellin and SPI-1 recognition, as infections with *S*. Typhimurium mutants lacking flagellin or SPI-1 almost completely prevented pyroptosis induction (Jesenberger et al., 2000, Lightfield et al., 2008, Miao et al., 2006). However, most of these studies investigated the cellular response to infection only within the first 2 hours post-infection. One report that investigated later time points (4 – 6.5 hours post-infection), showed that macrophages undergo a *S*. Typhimurium induced caspase-1 independent cell death with delayed kinetics and apoptotic features (Jesenberger et al., 2000). These findings strengthen the hypothesis that a functional backup system exists, which ensures cell death induction in infected macrophages independent of pyroptosis.

The roles of necroptosis and apoptosis within the immune response to intracellular infections, including *S*. Typhimurium, are less well understood and controversial (Jorgensen et al., 2017, Lamkanfi and Dixit, 2010). Overall, published *in vivo* data suggests no essential primary role for necroptosis against bacterial infections (Jorgensen et al., 2017). RIPK3 deficient mice and WT mice were similarly susceptible to various bacterial pathogens, including *S*. Typhimurium SL1344 (Robinson et al., 2012). In contrast, apoptotic host cell death was implicated to limit the replication and dissemination of several bacterial pathogens (Lamkanfi and Dixit, 2010). While induction of the intrinsic apoptotic pathway was shown to restrict the replication of *Legionella pneumophilia* (Nogueira et al., 2009) and pneumococci (Marriott et al., 2005), the extrinsic apoptotic pathway is triggered by *Pseudomonas aeruginosa* (Grassme et al.,

2000) and *Helicobacter pylori* infections (Jones et al., 2002). *S*. Typhimurium was suggested to induce apoptosis by interfering with the NF-κB and MAPK signalling cascades, which are important host survival pathways (Collier-Hyams et al., 2002). Furthermore, studies indicated that *S*. Typhimurium initiates intrinsic or extrinsic apoptosis via various effector proteins, including SipA (McIntosh et al., 2017), SipB, SipD, PrgH (Lin et al., 2021) or SpvB (Browne et al., 2002, Kurita et al., 2003). Additionally, previous findings revealed that *S*. Typhimurium infection induces caspase-8 recruitment to the inflammasome followed by proteolytic activation and IL-1β production (Man et al., 2013, Man et al., 2014b).

In vivo and *in vitro* studies using single knockout mice began to unravel the roles of different caspases in health and disease, including intracellular bacterial infections. However, potential overlapping and compensatory functions of caspases and PCD pathways are not well understood as multiple knockout mice models, lacking several caspases or types of PCD, have not been investigated extensively. The aims of this chapter are to determine the relative importance of diverse PCD initiators and effectors for immunity against *S*. Typhimurium infections and to investigate the ability of different PCD pathways to compensate for each other. To analyse this, we used sophisticated novel mouse strains deficient for different combinations of PCD mediators and infected them systemically with growth attenuated *S*. Typhimurium strains. This *in vivo* infection model allowed us to investigate innate immune mechanisms essential for *S*. Typhimurium control as well as T cell mediated mechanisms important for infection clearance (Benoun et al., 2018, Kupz et al., 2014, Kupz et al., 2012, Kupz et al., 2013). Furthermore, we established and optimised highly sensitive assays to measure cell death kinetics of BMDMs via the incorporation of PI and to quantify the intracellular burden of infected cells *in vitro*. The established high throughput approaches provide real-time resolution of cell death kinetics. Additionally, we gained insights into the correlation between the induction of PCD and intracellular bacterial replication. With these findings we aim to answer unexplored key scientific questions by deciphering the complex network of PCD and its importance as host defence mechanism against intracellular infection.

3.2 Results

3.2.1 Control and clearance of *Salmonella* Typhimurium infections in the absence of pyroptosis *in vivo*

3.2.1.1 Caspase-12 is not important for control and clearance of *Salmonella* Typhimurium infection *in vivo*

We methodically investigated which initiators or effectors of PCD compensate in the absence of caspases-1 and -11 and ensure *S*. Typhimurium control and clearance *in vivo*. Firstly, we analysed if caspase-12 is involved in orchestrating the induction of PCD during caspase-1 and -11 deficiency.

Caspase-12 was characterised as additional member of the family of inflammatory caspases, because of its chromosomal co-localization and high amino acid sequence similarity with caspase-1 and -11 (Lamkanfi et al., 2004). However, the functions of caspase-12 in inflammation, immunity against intracellular infections and PCD regulation are less well understood and published findings are controversial. It was shown that caspase-12 expression is upregulated by inflammatory stimuli (Kalai et al., 2003), including LPS and IFN-γ, which implies a potential role of caspase-12 during bacterial infections and sepsis. Additionally, it has been reported that caspase-12 negatively regulates pyroptosis and inflammatory responses and that *Casp12*$^{-/-}$ mice are more resistant to *Listeria monocytogenes* infections and sepsis (Saleh et al., 2006). Interestingly, human individuals of Asian and Caucasian descent express an enzymatically inactive truncated version of caspase-12 due to a premature stop codon mutation (Hermel and Klapstein, 2011, Saleh et al., 2004). Only around 20 % of African people harbour the functional full-length protein, which is associated with increased susceptibility to sepsis and decreased responses to LPS (Saleh et al., 2004). These findings were challenged by another study, which questioned the role of caspase-12 as inhibitor of caspase-1 (Vande Walle et al.,

2016). Whereas some studies suggested a role for caspase-12 in ER stress induced apoptosis (Jimbo et al., 2003, Nakagawa and Yuan, 2000, Nakagawa et al., 2000), others could not verify this (Kalai et al., 2003, Obeng and Boise, 2005, Salvamoser et al., 2019). Potential overlapping functions of caspases-1 and -11 could have hindered the identification of the role of caspase-12 in bacterial infections.

To analyse the possible compensatory role of caspase-12 for *S*. Typhimurium control in the absence of inflammatory caspases-1 and -11 *in vivo*, we infected caspases-1, -11 and -12 deficient mice (Salvamoser et al., 2019) with 200 CFU of the growth attenuated *S*. Typhimurium strain BRD509. The bacterial titers in liver and spleen were analysed 3 weeks post-infection, which represents the peak of infection in WT mice. *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice, unable to induce inflammatory caspase dependent pyroptosis, had slightly increased bacterial titers in liver and spleen compared to WT mice 3 weeks post-infection (**Figure 3.1 A**). Of note, the deletion of caspase-12 in addition to caspase-1 and -11 did not further increase the bacterial titers in liver and spleen observed in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$ mice 3 weeks post-infection (as shown in **Figure 3.8**). Additionally, we evaluated the impact of systemic *S*. Typhimurium infection on the wellbeing of mice by monitoring their body weight, physical appearance and behaviour. We also determined the weights of livers and spleens at time of analysis, as pronounced hepatomegaly and splenomegaly are signs of infection severity. *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice did not show any physical signs of severe disease, as body weights over the first 3 weeks of infection (**Figure 3.1 B**) and relative organ weights at time of analysis (**Figure 3.1 C**) were comparable to those of infected WT mice.

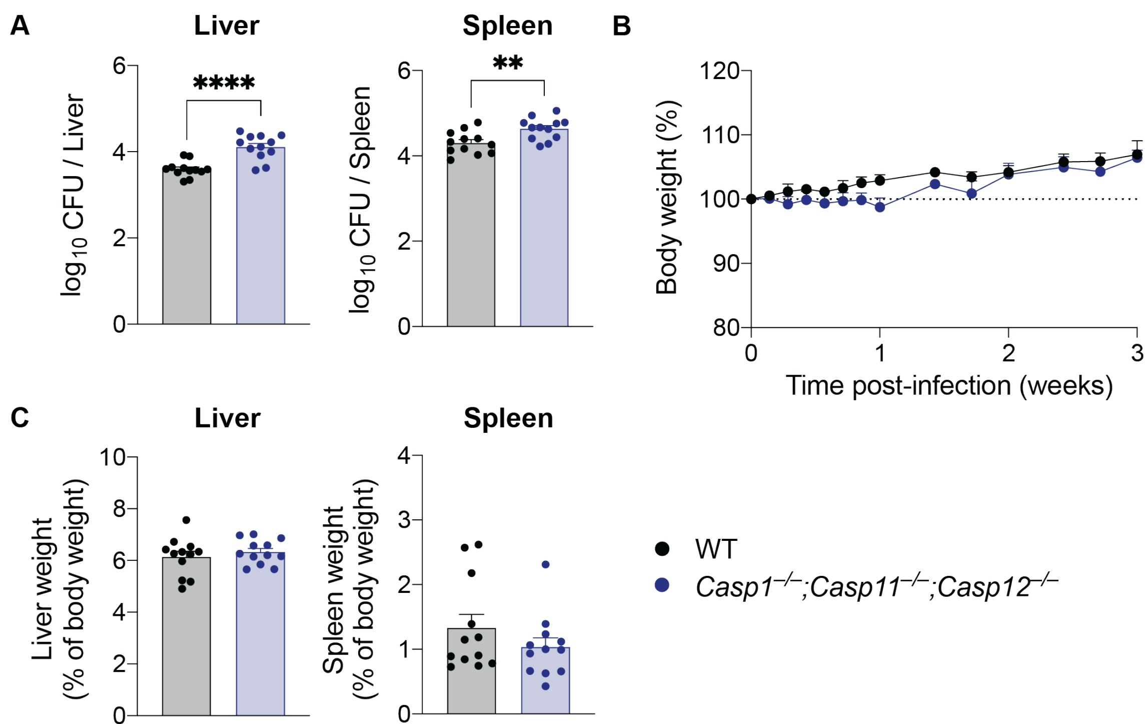


Figure 3.1: Combined loss of caspases-1, -11, and -12 leads to a slightly impaired *Salmonella* Typhimurium control *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ (blue) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen of WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed two to three times with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by unpaired Student's *t*-test (A and C) and calculated *p*-values are depicted (** $p < 0.01$; **** $p < 0.0001$).

To determine if the minor impairments in *S*. Typhimurium BRD509 control observed in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice 3 weeks post-infection affect bacterial clearance *in vivo*, we additionally investigated the bacterial titers of pyroptosis deficient mice 7 and 12 weeks post-infection. Consistent with previous findings (Kupz et al., 2014), bacterial titers in WT mice peaked around week 3 post-infection (**Figure 3.1 A**), followed by decreasing bacterial numbers in liver and spleen and clearance of infection within 12 weeks (**Figure 3.2 A**). *S*. Typhimurium numbers were initially slightly higher in the livers of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice compared to WT mice 7 weeks post-infection. However, focussing our analysis on 12 weeks post-infection no differences were detected between liver titers of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ and WT mice. Splenic bacterial titers were comparable between both groups 7 and 12 weeks post-infection. Furthermore, no differences in the body weights of infected WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice were detected (**Figure 3.2 B**). The constant weight gain indicates that infected mice did not develop a severe systemic infection. *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice had slightly increased relative liver weights 7 and 12 weeks post-infection compared to WT mice, while relative spleen weights were similar between groups (**Figure 3.2 C**). This observation reflects the findings obtained from bacterial titers in liver and spleen (**Figure 3.2 A**). The combined deletion of inflammatory caspases-1, -11 and -12 neither resulted in a further increase of bacterial titers in liver and spleen 7 and 12 weeks post-infection nor prolonged the bacterial clearance phase in comparison to *Casp1*$^{-/-}$*;Casp11*$^{-/-}$ mice (Doerflinger et al., 2020).

Taken together, we conclude that caspase-12 plays no important compensatory role in the absence of caspases-1 and -11 during *S*. Typhimurium control and clearance *in vivo*. These results extend on the findings that *S*. Typhimurium infections can be controlled without the induction of pyroptosis by inflammatory caspases-1 and -11 and imply the existence of an intricate backup system *in vivo*, that compensates for the loss of pyroptosis and ensures immunity against *S*. Typhimurium independent of caspase-12.

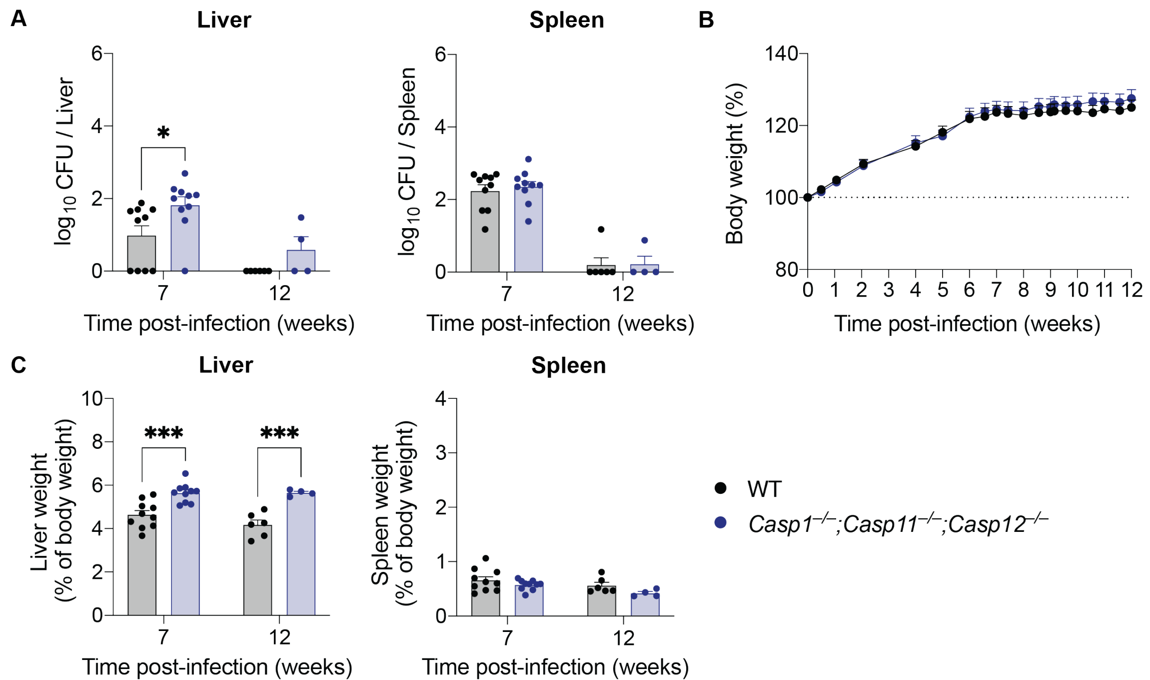


Figure 3.2: Combined loss of caspases-1, -11, and -12 does not prolong *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ (blue) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen of WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice were determined 7 and 12 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed one to three times with each experimental group including $n \geq 3$. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (A and C) and calculated *p*-values are depicted (* $p < 0.05$; *** $p < 0.001$).

3.2.1.2 Pyroptosis evading *Salmonella* Typhimurium strains are controlled *in vivo*

The redundancy of the PCD system most likely developed during co-evolution with infectious microorganisms, including intracellular pathogens, which have developed multiple strategies to manipulate host cells and prevent immune responses. *S*. Typhimurium harbours a wide range of effector molecules, including SopB and SpvC, that have been shown to interfere with PCD processes (Wemyss and Pearson, 2019). Besides that, intracellular *S*. Typhimurium bacteria avoid detection via PRRs and subsequent induction of pyroptosis by downregulation of PAMPs that are not essential within the cell, such as the T3SS encoded by SPI-1 or flagellin (Cirillo et al., 1998, Cummings et al., 2006, Galan, 1999, Galan and Collmer, 1999, Galan and Curtiss, 1989, Galan and Zhou, 2000, Hensel et al., 1998, Lai et al., 2013, Miao et al., 2010a, Mills et al., 1995, Ochman et al., 1996, Shea et al., 1996). Therefore, additional pyroptosis independent immune mechanisms may have evolutionary developed to ensure intracellular infection control and clearance *in vivo*.

To investigate if the proposed functional backup system counteracts pathogen evasion of pyroptosis and thereby ensures infection control *in vivo*, we infected WT mice with 200 CFU of the growth attenuated *S*. Typhimurium strains SL1344 *ΔaroAΔinvA* and SL1344 *ΔaroAΔfliCΔfljB*, which lack a functional SPI-1 encoded T3SS or flagellin respectively. We confirmed that SPI-1 encoded T3SS and flagellin mutant *S*. Typhimurium strains prevent pyroptosis induction *in vitro* (shown in **Figure 3.13 A**). WT mice infected with SL1344 *ΔaroAΔinvA* had slightly higher bacterial liver titers 3 weeks post-infection, while spleen titers were comparable to the infection with the growth attenuated strain SL1344 *ΔaroA* (**Figure 3.3 A**). The body weights of these two groups were similar throughout the experiment (**Figure 3.3 B**). Surprisingly, SL1344 *ΔaroAΔinvA* infected mice showed a less pronounced hepato- and splenomegaly, indicated by slightly lower relative liver and spleen weights at the peak of infection (**Figure 3.3 C**). This is

probably due to the lack of a functional SPI-1 encoded T3SS, which is required to inject bacterial effectors into host cells for host cell manipulation and infection of non-phagocytic cells thereby inducing cellular dysfunctions and inflammation in liver and spleen. Similarly, no differences in bacterial titers, mice body weights and relative organ weights were detected 3 weeks post-infection in WT mice infected with the non-flagellated *S*. Typhimurium strain SL1344 *ΔaroAΔfliCΔfljB* (**Figure 3.3 D – F**).

In summary, these findings indicate that systemic infections with *S*. Typhimurium mutant strains that do not trigger pyroptosis to the same extent than the WT strain are controlled without major impairments *in vivo*. This provides further evidence for the presences of a functional backup system that ensures infection control even if pyroptosis is evaded by *S*. Typhimurium.

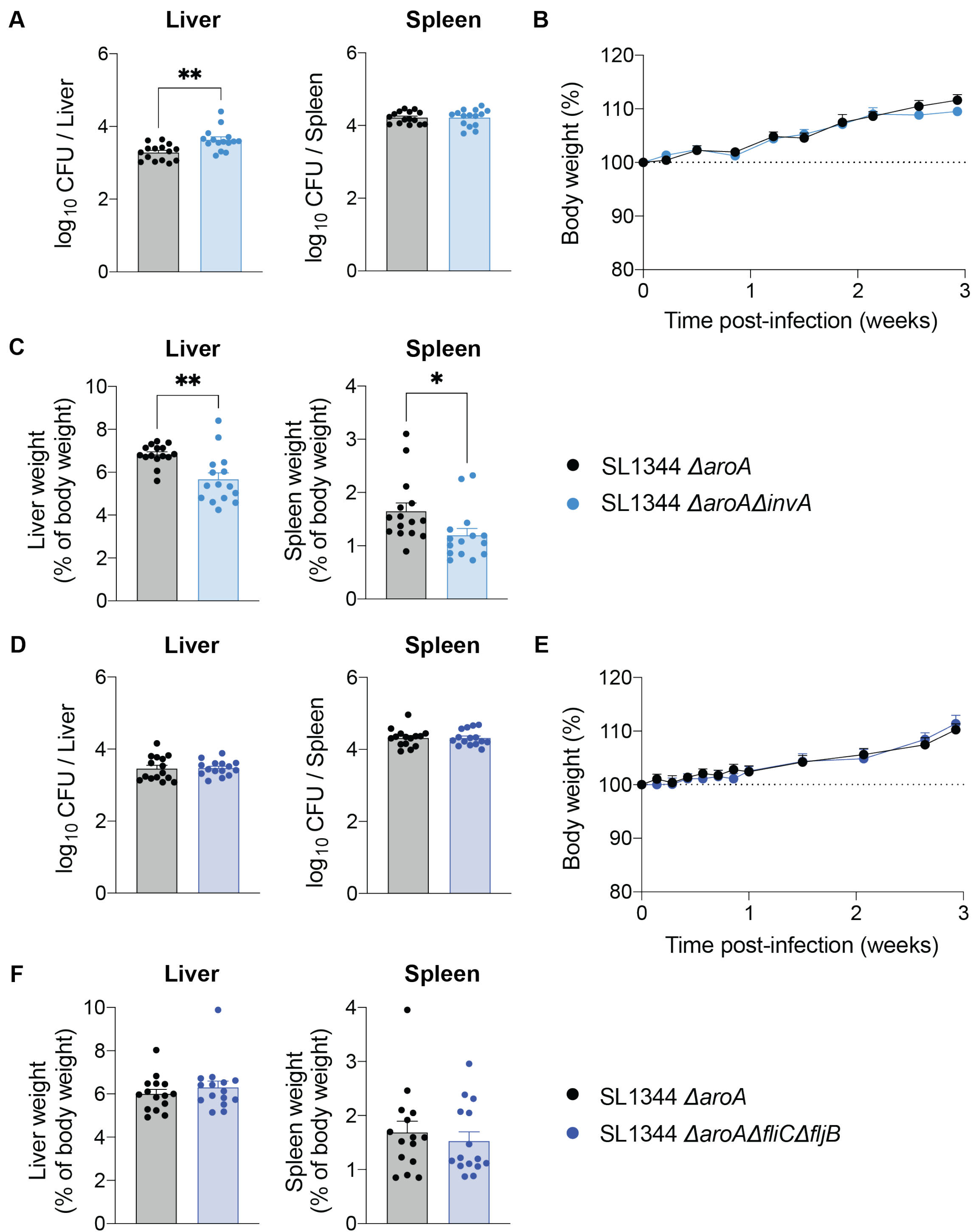

A
Liver
Spleen
log10 CFU / Liver
log10 CFU / Spleen
**
B
Body weight (%)
Time post-infection (weeks)
C
Liver
Spleen
Liver weight (% of body weight)
Spleen weight (% of body weight)
**
*
SL1344 ΔaroA
SL1344 ΔaroAΔinvA
D
Liver
Spleen
log10 CFU / Liver
log10 CFU / Spleen
E
Body weight (%)
Time post-infection (weeks)
F
Liver
Spleen
Liver weight (% of body weight)
Spleen weight (% of body weight)
SL1344 ΔaroA
SL1344 ΔaroAΔfliCΔfljB

Figure 3.3: Pyroptosis evading *Salmonella* Typhimurium strains are controlled without major impairments *in vivo*.
Wild-type mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain SL1344 *ΔaroA* (depicted in grey) or the mutant strains SL1344 *ΔaroAΔinvA* (light blue, A – C) and SL1344 *ΔaroAΔfliCΔfljB* (dark blue, D – F) unable to induce pyroptosis. (A and D) Bacterial titers in the liver and spleen were determined 3 weeks post-infection. (B and E) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C and F) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed three times with each experimental group including n = 5. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by unpaired Student's *t*-test (A, C, D and F) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$).

3.2.2 Necroptosis is not essential for control and clearance of *Salmonella* Typhimurium infection *in vivo*

We investigated if necroptosis, which is besides pyroptosis another form of lytic PCD, contributes to *S*. Typhimurium control and clearance *in vivo* by acting as functional backup mechanism in the absence of pyroptosis. Therefore, mice deficient for the necroptotic initiator RIPK3 in addition to the inflammatory caspases-1, -11 and -12 were generated. We infected *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Ripk3*$^{-/-}$ mice with 200 CFU of the growth attenuated *S*. Typhimurium strain BRD509 and compared their ability to control and subsequently clear the infection to WT mice.

Focussing our analysis on the peak of infection, the combined absence of pyroptosis and necroptosis resulted in a moderately impaired infection control indicated by significantly increased bacterial titers in liver and spleen compared to WT mice 3 weeks post-infection (**Figure 3.4 A**). This was in line with increased relative liver and spleen weights of *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Ripk3*$^{-/-}$ mice (**Figure 3.4 C**). However, infected mice did not lose body weight (**Figure 3.4 B**) or showed physical or behavioural changes, which indicates that mice did not development severe disease.

The bacterial numbers in liver and spleen of *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Ripk3*$^{-/-}$ mice were slightly higher than in *Casp1*$^{-/-}$;*Casp11*$^{-/-}$ or *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$ mice 3 weeks post-infection (**Figure 3.8**). However, the observed difference was only statistically significant within the spleen.

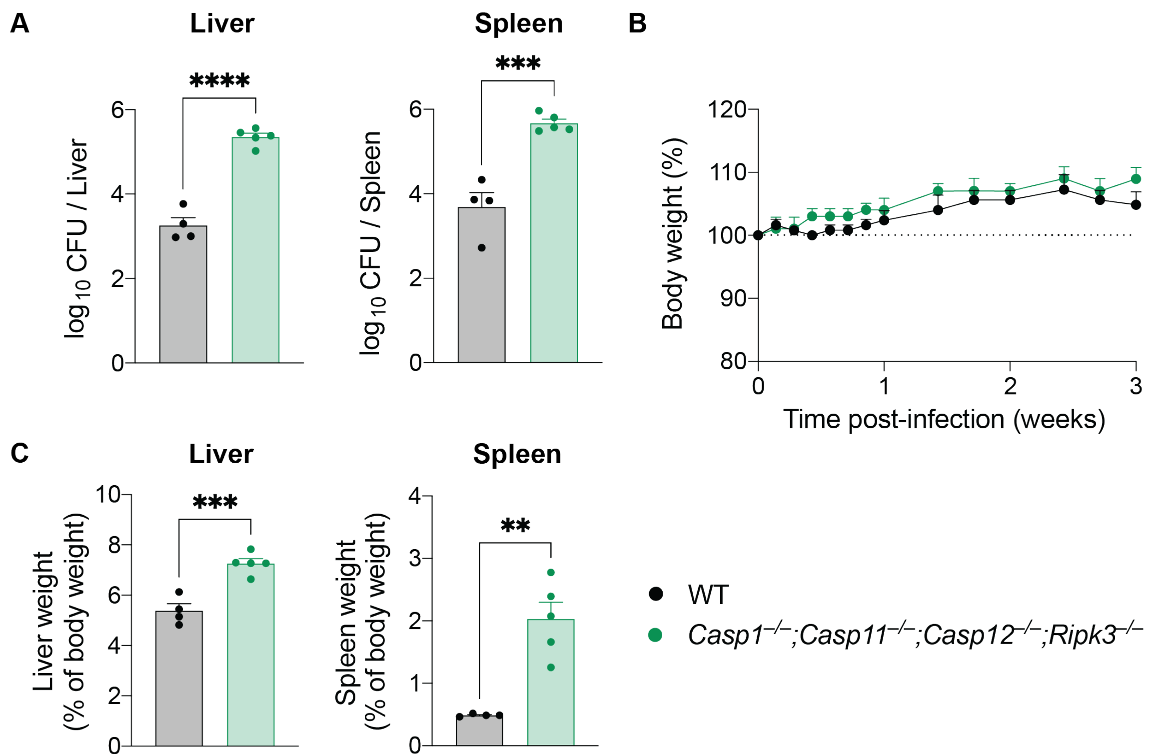


Figure 3.4: Combined caspase-1, -11, -12 and RIPK3 deficiency results in moderately impaired *Salmonella* Typhimurium control *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Ripk3*$^{-/-}$ (green) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed once with each experimental group including n ≥ 4. Data are expressed as mean + SEM. Statistically significant differences were determined by Student's *t*-test (A and C) and calculated *p*-values are depicted (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Next, we investigated if pyroptosis and necroptosis deficient mice were able to clear the infection by analysing later time points post-infection. *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Ripk3*$^{-/-}$ mice, despite the initial moderate impairments in *S*. Typhimurium control, cleared the infection similar to WT mice and no significant differences in bacterial titers were observed 7 and 12 weeks post-infection (**Figure 3.5 A**). This was also reflected in comparable body weights and organ weights of compared mice (**Figure 3.5 B**). The body weights of WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Ripk3*$^{-/-}$ mice increased similarly over the course of the infection, indicating no severe disease of the mice. Relative liver and spleen weights of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Ripk3*$^{-/-}$ mice were comparable to that of infected WT mice 7 and 12 weeks post-infection (**Figure 3.5 C**).

In conclusion, these results suggest that necroptosis mediated by RIPK3 might partially contribute to *S*. Typhimurium control in the absence of pyroptosis. However, the observation that mice lacking pyroptosis as well as necroptosis resolve the infection indicates that necroptosis is not essential for *S*. Typhimurium clearance. Therefore, additional mechanisms must be in place that compensate for the loss of pyroptosis and necroptosis and ensure clearance of *S*. Typhimurium infection *in vivo*.

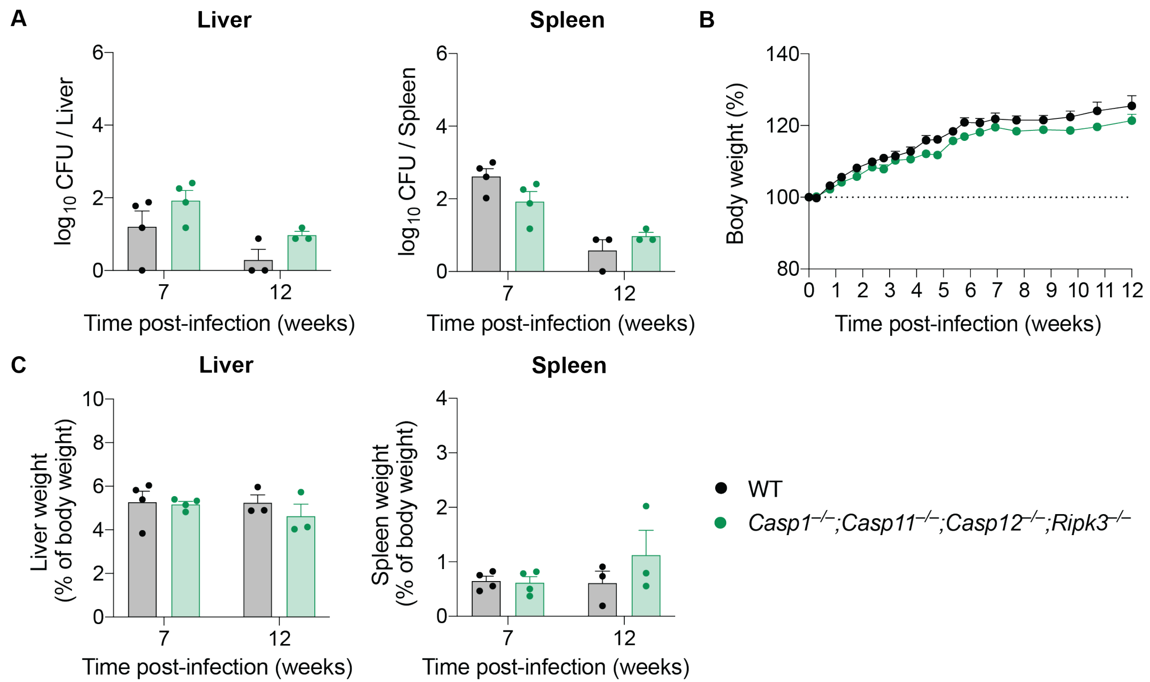


Figure 3.5: Combined caspase-1, -11, -12 and RIPK3 deficiency does not impact *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Ripk3*$^{-/-}$ (green) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 7 and 12 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed once with each experimental group including n ≥ 3. Data are expressed as mean + SEM. No statistically significant differences were determined by two-way ANOVA (A and C).

3.2.3 Combined absence of pyroptosis, necroptosis and extrinsic apoptosis severely impairs control and clearance of *Salmonella* Typhimurium infection *in vivo*

Previous findings revealed that *S*. Typhimurium infection induces caspase-8 recruitment to the inflammasome followed by proteolytic activation and IL-1β production (Man et al., 2013, Man et al., 2014b). Additionally, it was reported that caspase-8 initiates GSDMD cleavage to promote pyroptosis induction during *Yersinia* infection (Sarhan et al., 2018). Further studies, using *Legionella pneumophila* or *Francisella novicida* as Gram-negative bacterial infection models, reported that inflammasome activated caspase-8 triggers cell death in the absence of caspase-1 (Mascarenhas et al., 2017, Pierini et al., 2012, Schneider et al., 2017).

We therefore analysed if the *in vivo S*. Typhimurium control breaks down if death receptor-induced apoptosis mediated by caspase-8 is absent in addition to pyroptosis and necroptosis. Firstly, we infected *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice and analysed the *S*. Typhimurium control in the first 3 weeks post-infection. Bacterial titers in the liver of pyroptosis, necroptosis and extrinsic apoptosis deficient mice were significantly elevated compared to WT mice from week 2 post-infection onwards and drastically increased 3 weeks post-infection (**Figure 3.6 A**). Splenic bacterial titers of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice were also severely elevated 3 weeks post-infection. The drastically increase of bacterial numbers in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice indicates major impairments in *S*. Typhimurium control, while WT mice restrict bacterial growth resulting in the peak of titers around 3 weeks post-infection. Furthermore, the body weights of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice did fall under the initial body weight recorded at the start of the infection (**Figure 3.6 B**). In contrast to that, the body weights of WT mice remained stable. Relative liver weights were comparable between both groups with a trend towards enlarged

livers in PCD deficient mice 3 weeks post-infection (**Figure 3.6 C**). However, infected *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice developed a severe splenomegaly compared to WT mice, which was significant 3 weeks post-infection. These observations suggest that *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice develop uncontrolled *S*. Typhimurium infections. The increased liver and spleen sizes of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice most likely compensated partially for the overall weight loss.

Taken together, our findings indicate that the control of *S*. Typhimurium infection is heavily compromised in mice with combined deletion of pyroptosis, necroptosis and extrinsic apoptosis. This suggests an important role for caspase-8 mediated apoptosis during intracellular infections *in vivo*, although scaffolding functions of caspase-8 unrelated to cell death cannot be excluded.

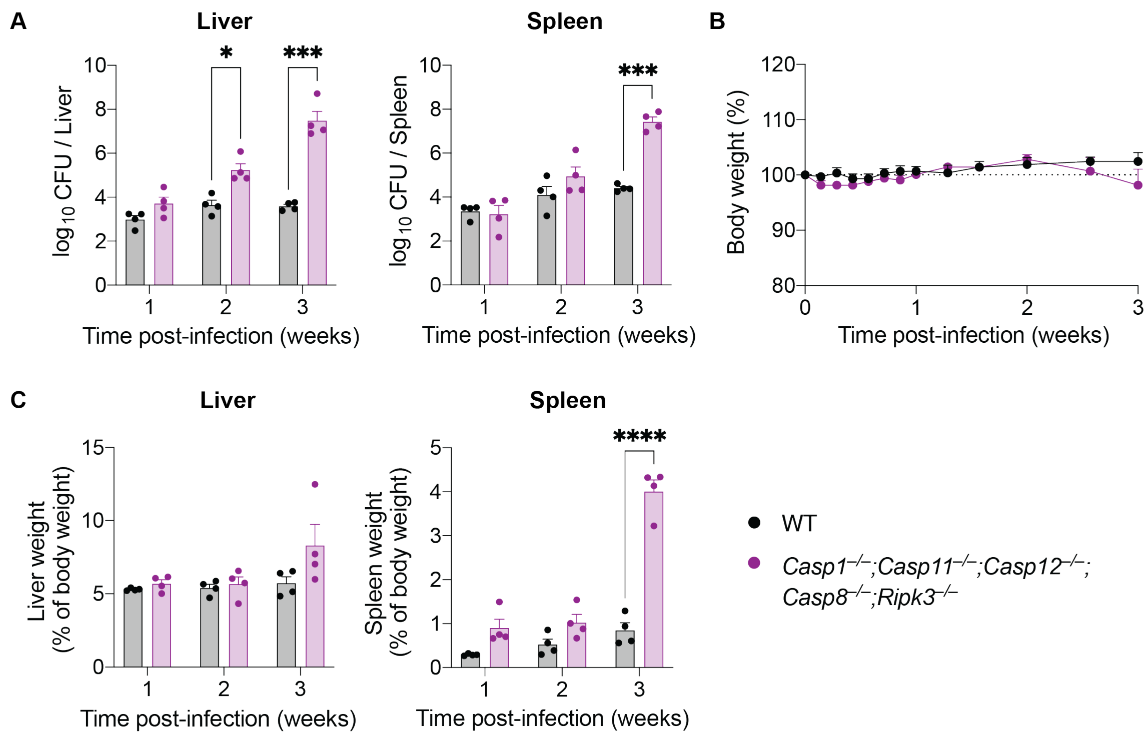


Figure 3.6: The combined loss of caspases-1, -11, -12, -8 and RIPK3 leads to impaired *Salmonella* Typhimurium control *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ (purple) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 1, 2 and 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed once with each experimental group including n = 4. Data are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (A, B and C) and calculated *p*-values are depicted (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$).

To determine if mice clear *S*. Typhimurium infections through pyroptosis, necroptosis and extrinsic apoptosis independent mechanisms despite the initial impaired infection control, we next infected *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$* mice and analysed the infection response past the 3 week time point.

Interestingly, infected *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$* mice developed severe disease, which was reflected in the overall physical appearance, behaviour and body weight loss and succumbed to infection between 4 – 5 weeks post-infection (**Figure 3.7 A**). Observed bacterial titers in liver and spleen at the time of sacrifice were significantly elevated compared to WT mice and further increased after the 3 week time point (**Figure 3.7 B**). Unrestricted *S*. Typhimurium replication and progression of the infection resulted in a significant loss of body weight starting between week 3 and 4 post-infection, while WT mice that clear the infection constantly gained weight (**Figure 3.7 C**). Additionally, *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$* mice developed severe hepato- and splenomegaly, which partly compensated for the actual amount of body weight loss (**Figure 3.7 D**).

These results indicate that mice are unable to clear *S*. Typhimurium infections in the combined absence of pyroptosis, necroptosis and extrinsic apoptosis. Additional remaining PCD mechanisms, such as intrinsic apoptosis, are not involved or efficient enough to restrict *S*. Typhimurium growth and ensure control and clearance of the infection.

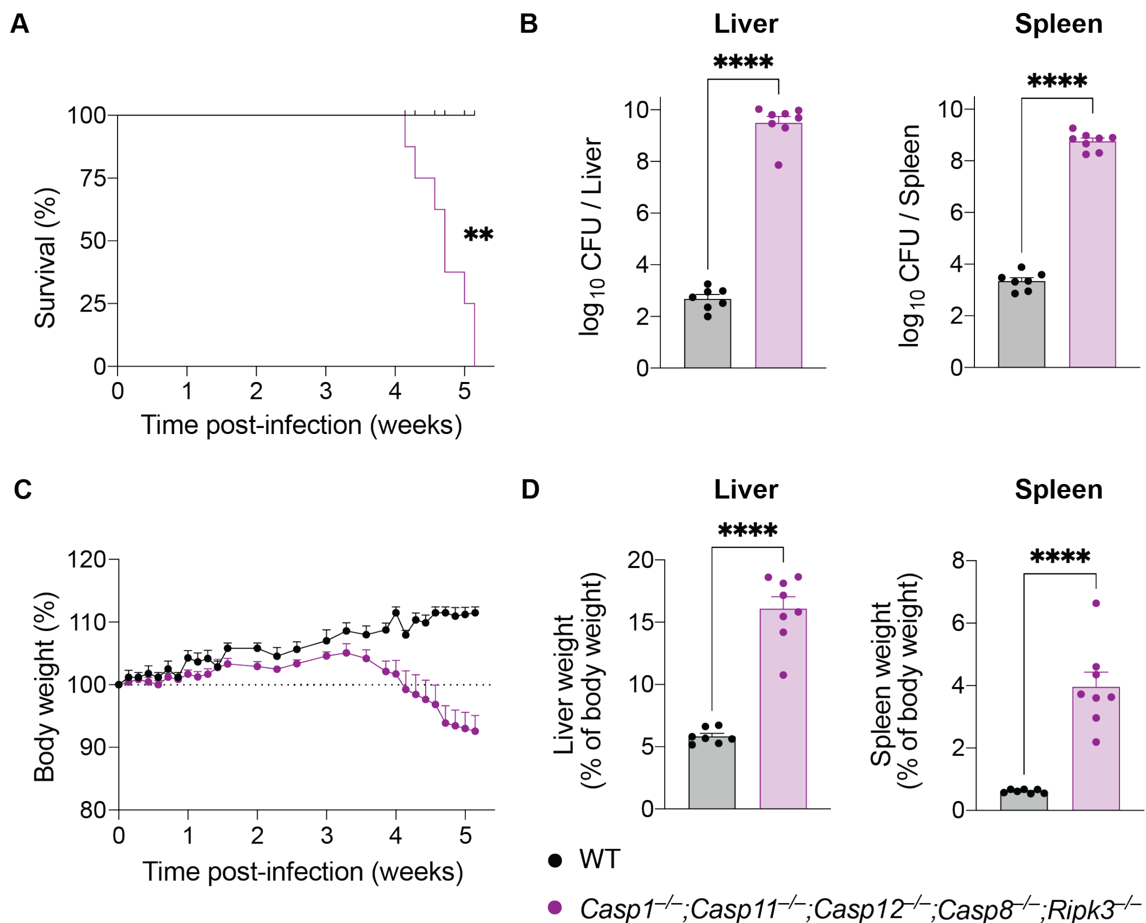


Figure 3.7: The combined loss of caspases-1, -11, -12, -8 and RIPK3 leads to impaired *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ (purple) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) The survival of infected mice was analysed based on body condition, appearance and behaviour. (B) Bacterial titers in the liver and spleen were determined at the time of sacrifice. (C) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (D) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of sacrifice. All experiments were performed twice with each experimental group including $n \geq 3$. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by log rank (Mantel cox) (A) or Student's *t*-test (B and D) and calculated *p*-values are depicted (** $p < 0.01$; **** $p < 0.0001$).

3.2.4 Flexible induction of different programmed cell death pathways ensures control of *Salmonella* Typhimurium infection *in vivo*

The generation of multiple knockout mouse strains, deficient for different combinations of caspases and RIPK3, unravelled the relative importance of pyroptosis, necroptosis and apoptosis for the control of systemic infections with growth attenuated *S*. Typhimurium. Minor impairments in *S*. Typhimurium control indicated by slightly elevated titers in liver and spleen were observed in pyroptosis deficient *Casp1*$^{-/-}$*;Casp11*$^{-/-}$ mice compared to WT mice 3 weeks post-infection (**Figure 3.8**). Similarly, mice lacking pyroptosis and necroptosis were able to control the intracellular infection. We also investigated the contribution of caspase-8 mediated apoptosis to *S*. Typhimurium control. As caspase-8 deficiency results in uncontrolled necroptosis and embryonic lethality (Alvarez-Diaz et al., 2016, Oberst et al., 2011), additional deletion of RIPK3 or MLKL is required to prevent this (Kaiser et al., 2011). We therefore infected *Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice unable to induce caspase-8 mediated apoptosis and necroptosis. Comparable with other mice lacking one or a combination of two PCD pathways, *Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice controlled *S*. Typhimurium infection without major impairments 3 weeks post-infection. In contrast, mice deficient for all three PCD pathways failed to control *S*. Typhimurium infections indicated by severely elevated bacterial titers in liver and spleen 3 weeks post-infection.

Collectively, these findings imply that the flexible induction of pyroptosis, apoptosis and necroptosis ensures the control of *S*. Typhimurium infections *in vivo*. The loss or evasion of one PCD pathway can be compensated by the activation of another type of PCD.

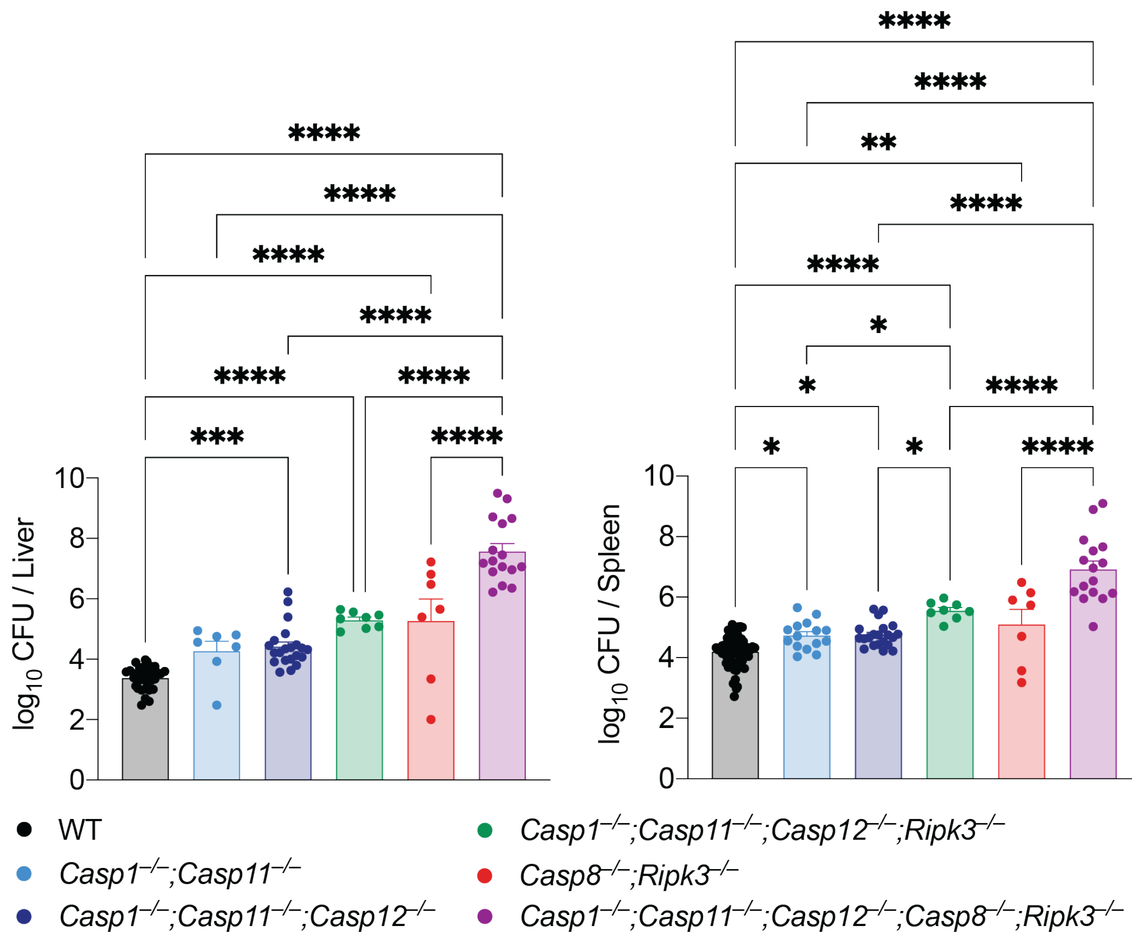


Figure 3.8: The combined loss of programmed cell death pathways leads to an impaired *Salmonella* Typhimurium control *in vivo*.
Wild-type (WT; depicted in black), *Casp1$^{-/-}$;Casp11$^{-/-}$* (light blue), *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$* (dark blue), *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Ripk3$^{-/-}$* (green), *Casp8$^{-/-}$;Ripk3$^{-/-}$* (red) and *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$* (purple) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. Bacterial titers in the liver and spleen were determined 3 weeks post-infection. All experiments were performed at least twice with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by one-way ANOVA and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Data obtained within this study were pooled with data generated by Paul Whitney and Ranja Salvamoser.

3.2.5 Programmed cell death of hematopoietic cells is essential for control and clearance of *Salmonella* Typhimurium infection *in vivo*

Given the observed importance of PCD for control of clearance of *S*. Typhimurium infections we next investigated which cell subset requires PCD to limit bacterial replication *in vivo*. To analyse the importance of PCD of hematopoietic cells and non-hematopoietic cells for restriction of bacterial replication and *S*. Typhimurium control, we generated bone marrow chimeric mice, in which the hematopoietic cell subset is deficient for pyroptosis, extrinsic apoptosis and necroptosis.

The hematopoietic system of WT mice was replaced with cells from *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice and these chimeric mice were infected with 200 CFU of *S*. Typhimurium BRD509. The bacterial titers in liver and spleen were investigated 3 weeks post-infection. The bacterial numbers were drastically elevated in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ chimeric mice compared to chimeric mice that were reconstituted with WT bone marrow cells (**Figure 3.9 A**). Noteworthy, this observed increase of bacterial titers was comparable to that of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice, which lack caspases-1, -11, -12, -8 and RIPK3 in all of their cells (**Figure 3.6 A**). Additionally, the body weights of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ chimeric mice slightly decreased towards 3 weeks post-infection (**Figure 3.9 B**), which was in part compensated by enlarged livers and spleens of infected chimeric mice (**Figure 3.9 C**).

These findings reveal that induction of PCD in the immune cell compartment is essential to control *S*. Typhimurium infections *in vivo*. The hematopoietic system includes phagocytic cells, such as macrophages, which have been reported to be one of the major replicative sites of *S*. Typhimurium. Our results imply that the restriction of bacterial replication most likely fails because phagocytes cannot undergo PCD to purge intracellular bacteria.

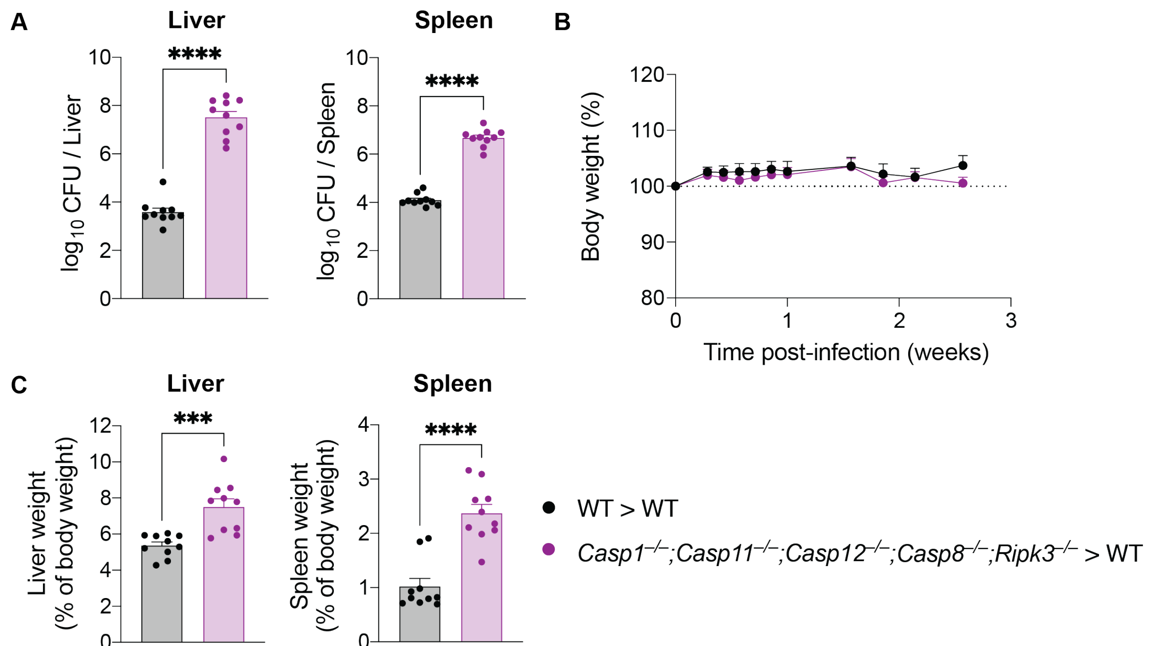


Figure 3.9: The combined loss of caspases-1, -11, -12, -8 and RIPK3 in hematopoietic cells leads to impaired *Salmonella* Typhimurium control *in vivo*.
Bone marrow chimeric mice of the indicated genotypes (Wild-type (WT) depicted in black and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ depicted in purple) were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed twice with each experimental group including n = 5. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by unpaired Student's *t*-test (A and C) and calculated *p*-values are depicted (*** $p < 0.001$; **** $p < 0.0001$).

3.2.6 Establishment of *in vitro* assays to disentangle the mechanisms of programmed cell death regulation during *Salmonella* Typhimurium infection

The observation that a functional backup system between pyroptosis, necroptosis and apoptosis ensures the control of *S*. Typhimurium infections *in vivo* raised the question how this flexible network is orchestrated. To study the role of individual PCD pathways, the underlying mechanisms of their regulation and cell death kinetics during intracellular infection in more detail, a reliable *in vitro* model is needed. Moreover, potential connections between distinct PCD pathways and the proposed involvement of effector molecules in multiple pathways require further investigation. Due to the importance of macrophages in *S*. Typhimurium control and clearance, we need a better understanding of the immune responses of primary macrophages following intracellular infection.

3.2.6.1 Generation and characterisation of bone marrow-derived macrophages

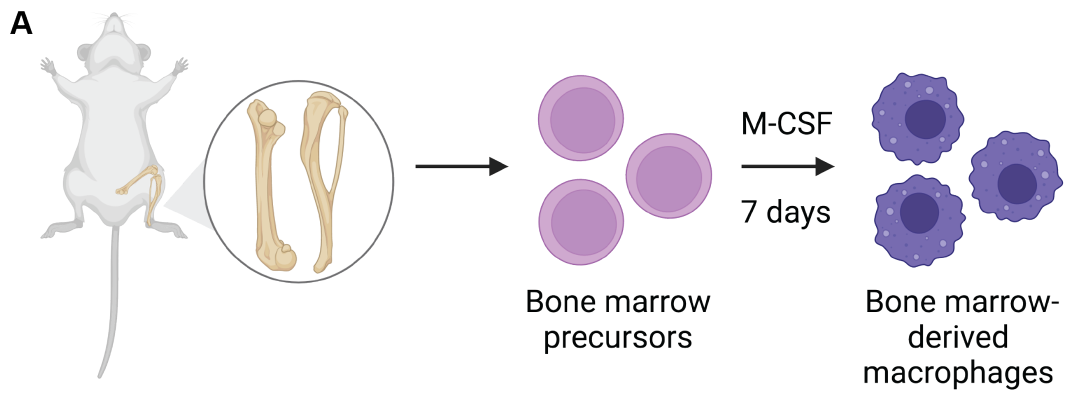
In the presence of M-CSF, bone marrow (BM) progenitor cells develop into BMDMs (Hamilton et al., 2014, Manzanero, 2012, Rios et al., 2017, Weischenfeldt and Porse, 2008). M-CSF is a cytokine and growth factor that affects the proliferation and differentiation of macrophages and is essential for their survival and function (Stanley et al., 1978, Stanley et al., 1983). The mouse fibroblast cell line L929 produces M-CSF and their culture supernatant is widely used as component of BMDM differentiation media (Weischenfeldt and Porse, 2008).

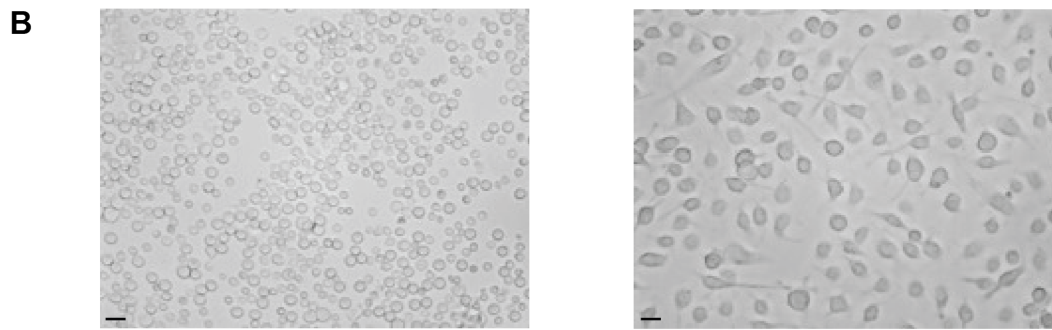
To generate BMDMs, BM precursors were incubated with 15 % L929 supernatant conditioned media for 7 days (**Figure 3.10 A**). The differentiation of BMDMs was evaluated based on changes in morphology and surface marker expression. We

analysed the morphology of BM-derived cells during incubation with L929 supernatant using light microscopy. Changes in cell appearance and adherence were observed after 3 days of cell culture and manifested towards day 7 (**Figure 3.10 B**). During differentiation, the round, non-adherent BM precursor cells developed into a monolayer of elongated, spindle-like shaped BMDMs that strongly adhered to non-treated tissue culture petri dishes. This validated that the generated cells had the expected morphology of macrophages.

Mature BMDMs and their activation are characterised by the expression of specific surface antigens which can be identified by flow cytometry analysis (Weischenfeldt and Porse, 2008, Ying et al., 2013). To validate the differentiation of BM precursor cells into BMDMs, we therefore analysed the expression of classical macrophage markers F4/80 and CD11b (Wynn et al., 2013) on generated cells after 7 days of incubation. Additionally, expression levels of MHC-II molecules, which can be found on professional APCs critical for the initiation of the antigen-specific immune response, and of the activation marker CD83 were measured. We also determined the expression of the costimulatory molecules CD80 and CD86, typically expressed by APCs and important for T cell stimulation. Cultured cells were F4/80$^+$CD11b$^+$ indicating the purity of mature BMDMs (**Figure 3.10 C**). Differentiated macrophages expressed low levels of MHC-II molecules and CD83, indicating that cells were not highly activated under basal conditions. Besides that, the constitutively expressed costimulatory molecules CD80 and CD86 were found on the surface of the cells.

Taken together, these results demonstrate that the cell culture protocol resulted in the differentiation of BM precursor cells into a pure population of mature BMDMs, that can be used in subsequent assays as *in vitro* model to study PCD in the context of *S.* Typhimurium infection.


A
M-CSF
7 days
Bone marrow precursors
Bone marrow-derived macrophages


B
Day 0
Day 7

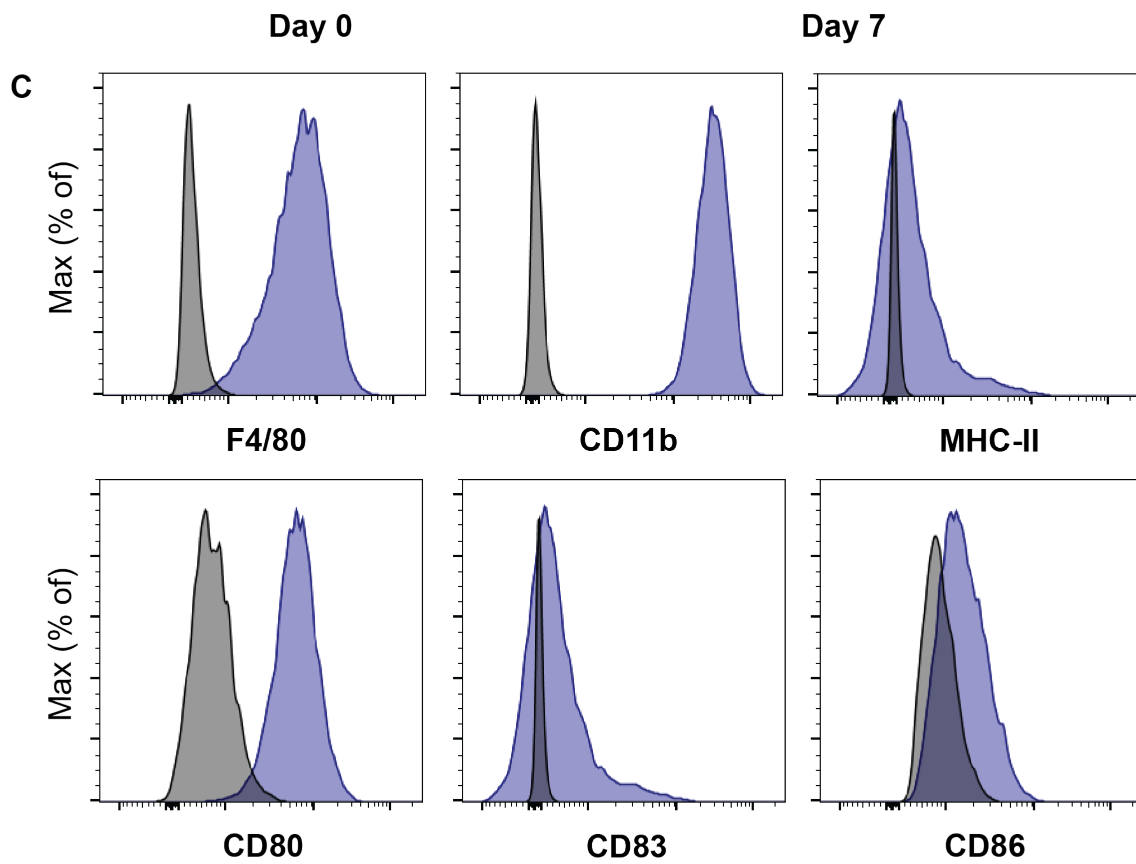

C
Max (% of)
F4/80
CD11b
MHC-II
Max (% of)
CD80
CD83
CD86

Figure 3.10: Differentiation and characterisation of bone marrow-derived macrophages.

(A) Bone marrow precursors were harvested from murine femurs and tibias and differentiated into bone marrow-derived macrophages (BMDMs) in the presence of macrophage colony-stimulating factor (M-CSF). (B) Brightfield microscopy of bone marrow progenitors on day 0 and differentiated BMDMs on day 7 of incubation with 15 % L929-conditioned media (scale bar: 20 µm). Representative images of the cell culture are depicted. (C) The expression of the macrophage surface and activation markers F4/80, CD11b, major histocompatibility complex (MHC)-II, CD80, CD83 and CD86 on viable BMDMs was analysed by flow cytometry after 7 days of incubation with 15 % L929-conditioned media. Representative histograms of the cell surface marker expression are depicted.

3.2.6.2 Optimisation of cell death kinetics measurements via incorporation of propidium iodide

To study the regulation and induction of PCD following *S*. Typhimurium infection, we developed and optimised an *in vitro* assay to analyse cell death kinetics in real-time measured by the PI fluorescence using the microplate reader CLARIOstar Plus. PI is a fluorescent intercalating agent used to detect dead cells. PCD leads to the loss of plasma membrane integrity in dying cells allowing PI, which is normally cell-impermeant, to bind to intracellular DNA and accumulate.
To determine the sensitivity of the assay and to analyse how cell death kinetics are influenced by the number of bacteria the cells are exposed to, we first infected BMDMs with different MOI of the *S*. Typhimurium strain BRD509. Therefore, PI was added to the culture medium of infected BMDMs and cellular PI fluorescence accumulation measured over time. *S*. Typhimurium BRD509 infection of WT BMDMs resulted in cellular PI staining indicating that infected cells lose their plasma membrane potential and incorporate PI within the first hours post-infection (**Figure 3.11 A**). Infections with MOI 10 and 1 resulted in 80 % and 60 % PI^{+} cells while a MOI of 0.1 only led to around 5 % PI^{+} cells. Infection with higher MOI correlated with an earlier and higher PI signal overall. Uninfected cells (MOI 0) remained PI impermeable during the analysed time interval.

We investigated the influence of extracellular bacterial growth on cell death induction over time. Therefore, the antibiotic gentamicin was added to the cell culture media after 1 hour of infection to prevent subsequent extracellular bacterial growth (**Figure 3.11 B**). Additionally, different concentrations of gentamicin were tested to determine potential cytotoxic effects of the antibiotic towards BMDMs. The amount of PI^{+} cells after infection of BMDMs with MOI 10 decreased from 80 % without gentamicin in the media to 40 – 50 % PI^{+} cells with increasing gentamicin concentration. The addition of gentamicin resulted in a decrease from 60 % to 5 % PI^{+} cells when BMDMs were infected with a MOI of 1. The small proportion of cell death observed in infection conditions with MOI 0.1 was completely prevented with the addition of gentamicin. The addition of

gentamicin (10 – 100 µg/mL) to uninfected BMDMs (MOI 0) did not result in any PI uptake of cells.

These results demonstrate that we developed a highly sensitive *in vitro* approach to measure the cell death kinetics of infected BMDMs via the intracellular accumulation of PI in real-time. The established approach allows us to resolve the involvement and kinetics of different PCD pathways during *S*. Typhimurium infection in great detail. This is an enormous advantage over other conventional methods used to detect cell death, including LDH assay or FACS.

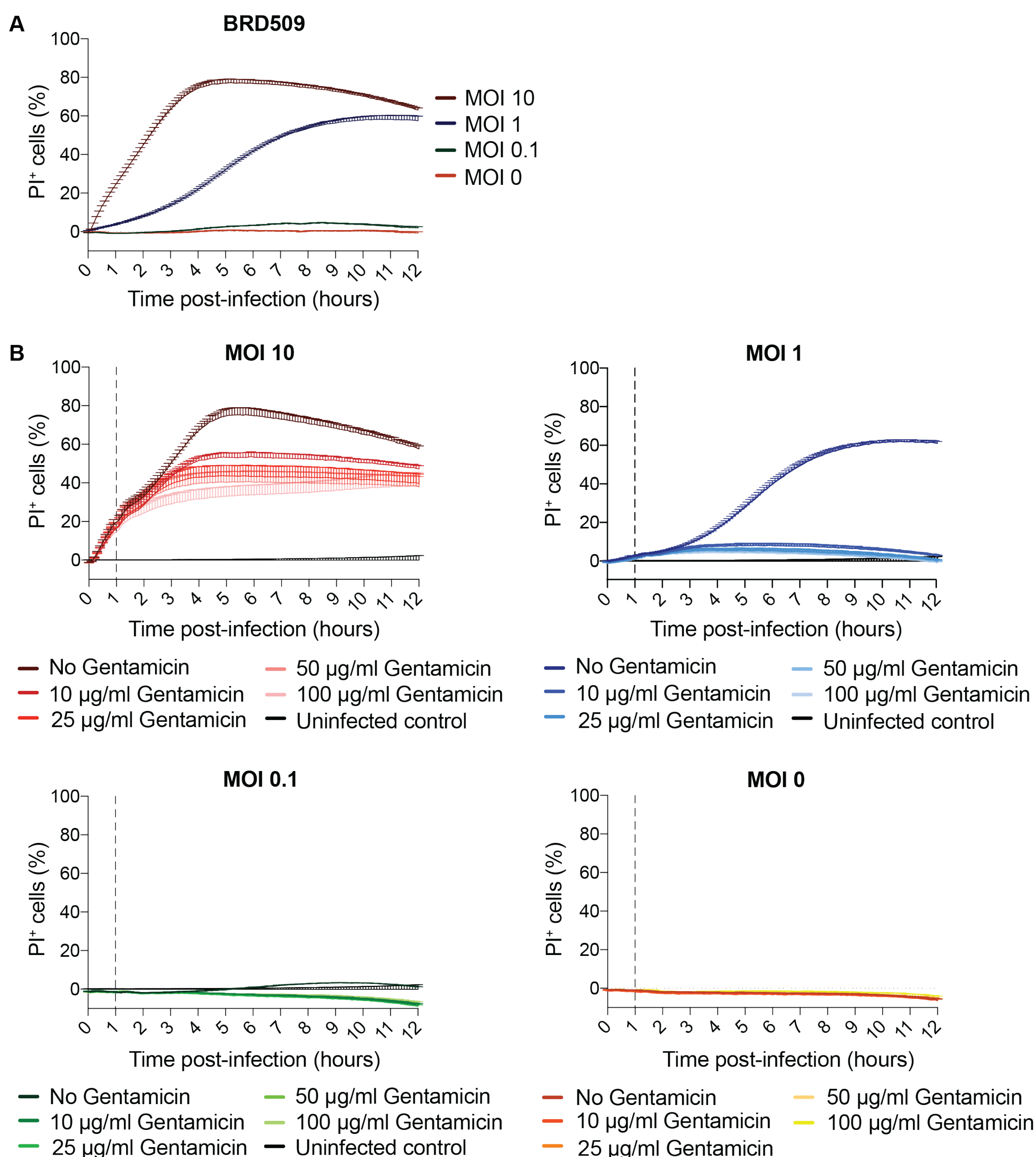


Figure 3.11: Cell death kinetics of *Salmonella* Typhimurium infected BMDMs.
Wild-type bone marrow-derived macrophages (BMDMs) were infected *in vitro* with the growth attenuated *Salmonella* Typhimurium strain BRD509 (MOI 0 – 10). (A) The incorporation of propidium iodide (PI; a marker of cell death) of infected BMDMs was measured over a time period of 12 hours post-infection. (B) Gentamicin (0 – 100 µg/mL) was added to BMDMs 1 hour post-infection (indicated by dotted line) to remove extracellular bacteria. The incorporation of PI of infected BMDMs was measured over a time period of 12 hours post-infection. All experiments were performed twice with ≥ 6 technical repeats. Data were pooled and are expressed as mean + SEM.

3.2.6.3 Optimisation of the quantification of intracellular bacteria

We established an *in vitro* gentamicin protection assay to analyse the influence of cell death mediators and PCD pathways on intracellular bacterial number, survival and replication of *S*. Typhimurium in macrophages. We compared different gentamicin exposure concentrations and durations to create a protocol that allows us to measure the intracellular bacterial burden of infected BMDMs *in vitro* and assures the inhibition of extracellular bacterial growth.

BMDMs were infected with *S*. Typhimurium BRD509 (MOI 10) for 1 hour and subsequently exposed to gentamicin (**Figure 3.12 A**). After 1 hour of infection around 1.25×10^6 cell associated bacteria and 5×10^5 extracellular bacteria were detected per well (**Figure 3.12 B**). Of note, this almost reflected the total number of 2×10^6 bacteria added per well for the infection of BMDMs. Intracellular and extracellular bacterial numbers exceeded the detection limit of 2×10^6 bacteria 2, 6 and 24 hours after infection in conditions where no gentamicin was added indicating uncontrolled replication when extracellular growth was not prevented. Similarly, the addition of gentamicin in a low dose (10 µg/mL) resulted in extracellular bacterial numbers beyond the detection limit 2 hours after infection. At later time points however, only very few bacteria could be detected in the supernatant suggesting that the low gentamicin concentration prevents extracellular replication over time. Intracellular bacterial numbers of around 1×10^5 were measured 2 and 6 hours after infection, which declined to very few viable bacteria at the 24 hour time point. The exposure to a high dose of gentamicin (100 µg/mL for 1 hour) resulted in no extracellular bacteria 2 hours after the infection. However, at later time points after gentamicin was removed bacterial numbers exceeded the detection limit. Intracellular bacterial numbers increased from around 5×10^4 to more than 2×10^6 bacteria between 2 and 24 hours after infection in this condition. The exposure to a high dose of gentamicin (100 µg/mL for 1 hour) followed by a low dose of gentamicin

(10 µg/mL for 22 hours) resulted in the successful inhibition of extracellular bacterial growth. Intracellular bacterial numbers around 5×10^4 were measured 2 and 6 hours after infection, which reduced towards the 24 hour time point.

The presented results demonstrate that optimised assay conditions allow quantification of the intracellular bacteria burden of *in vitro* infected cells while preventing extracellular bacterial growth. The exposure to 100 µg/mL gentamicin for 1 hour followed by 10 µg/mL gentamicin for 22 hours effectively inhibits extracellular bacterial growth after the infection.

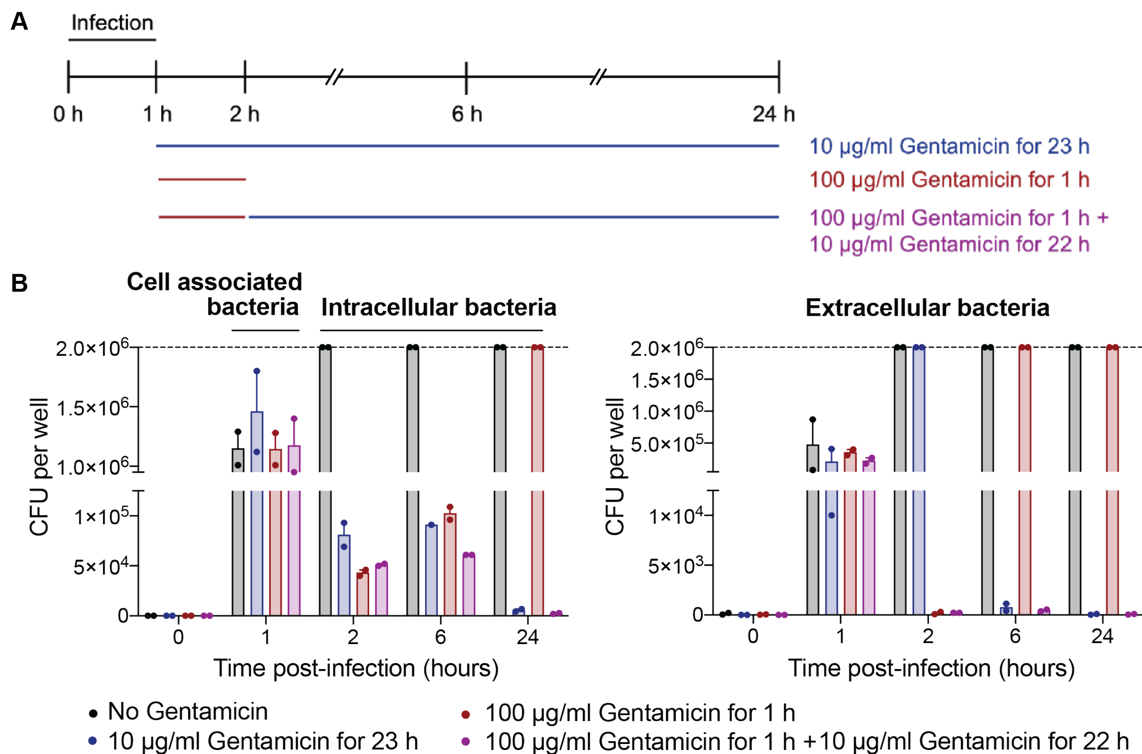


Figure 3.12: Intracellular bacterial burden of *Salmonella* Typhimurium infected BMDMs.

(A) Wild-type bone marrow-derived macrophages (BMDMs) were infected *in vitro* with the growth attenuated *Salmonella* Typhimurium strain BRD509 (MOI 10). Gentamicin was added to BMDMs 1 hour post-infection in different concentrations (10 – 100 µg/mL) to remove extracellular bacteria. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs as well as extracellular CFU per well were determined at the indicated time points post-infection. The dotted line indicates the maximal detection limit. The experiment was performed twice with one technical repeat. Data were pooled and are expressed as mean + SEM.

3.2.7 Macrophages undergo pyroptosis-independent cell death with delayed kinetics during *Salmonella* Typhimurium infection *in vitro*

S. Typhimurium infection of macrophages triggers the rapid induction of pyroptosis. The detection of intracellular bacterial PAMPs, such as components of the SPI-1 encoded T3SS and flagella, initiates inflammasome assembly and activation of caspase-1. Several studies showed that NLRC4 and caspase-1 deficient cells did not induce pyroptosis after *S*. Typhimurium infection *in vitro* (Jesenberger et al., 2000, Lara-Tejero et al., 2006, Lightfield et al., 2008, Mariathasan et al., 2004, Miao et al., 2006). However, our *in vivo* findings (**Figures 3.1 – 3.8**) suggested that caspase-8 dependent back-up mechanisms ensure the induction of PCD in the absence of pyroptosis.

To test if we could reproduce this *in vivo* finding with our newly established *in vitro* cell death assays, we first infected pyroptosis deficient *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs with *S*. Typhimurium SL1344 *in vitro*. In contrast to WT BMDMs, which induce pyroptotic cell death directly after *S*. Typhimurium infection with a MOI 10, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs died in an pyroptosis-independent manner with delayed kinetics starting between 3 – 4 hours post-infection (**Figure 3.13 A**). While approximately 50 % of WT BMDMs were PI^{+} 6 hours post-infection, only a small proportion of around 10 % of *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs underwent *S*. Typhimurium related cell death. Infections of BMDMs with MOI 50 led to an increase in the cell death rate to 70 % and 35 % respectively. The pyroptosis-independent cell death in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs was similarly observed with delayed kinetics starting between 2 – 3 hours post-infection. This delay on cell death might be explained by the induction of caspase-8 mediated apoptosis in line with the above-described *in vivo* results.

Additionally, we determined the intracellular bacterial numbers of infected WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs to analyse the role of pyroptosis on

S. Typhimurium survival and replication within infected BMDMs. *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$ BMDMs, unable to induce pyroptosis, harboured significantly higher amounts of bacteria 2 and 6 hours post-infection compared to WT BMDMs when infected with MOI 10 (**Figure 3.13 B**). Intracellular bacterial numbers increased between 2 and 6 hours post-infection indicating that *S*. Typhimurium replicated within macrophages. In contrast to that, bacterial titers in *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$ BMDMs infected with MOI 50 decreased over time, which could be explained by the observed increased cell death rate.

Taken together, we verified previous findings indicating that pyroptosis deficient BMDMs are protected from rapid *S*. Typhimurium induced cell death (Franchi et al., 2006, Mariathasan et al., 2004), but undergo cell death with delayed kinetics (Jesenberger et al., 2000). As *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$*;Casp8*$^{–/–}$*;Ripk3*$^{–/–}$ mice succumbed to *S*. Typhimurium infection, we speculate that this slower type of cell death might be caspase-8 dependent.

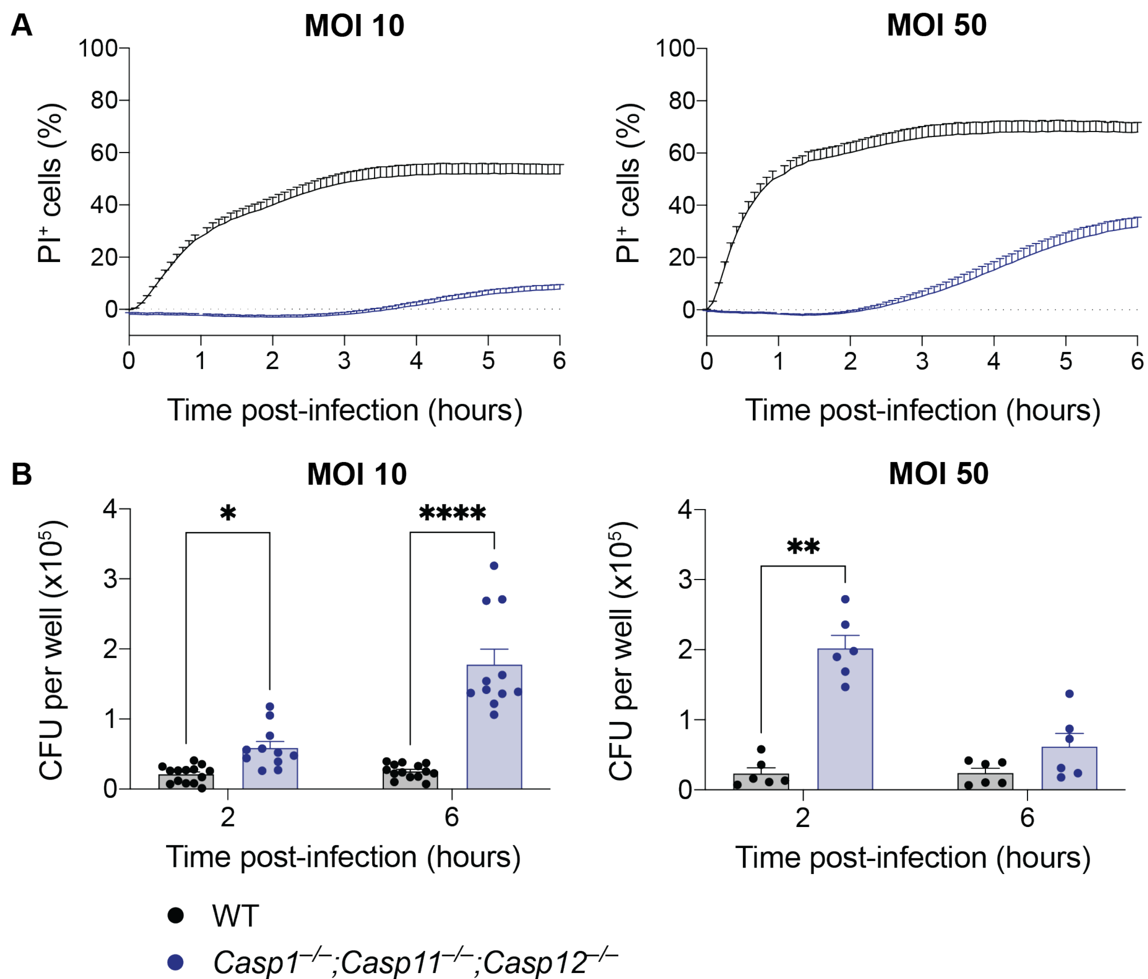


Figure 3.13: Pyroptosis deficient macrophages undergo *Salmonella* Typhimurium induced cell death with delayed kinetics.
(A and B) Wild-type (WT; depicted in black) and *Casp1⁻/⁻;Casp11⁻/⁻;Casp12⁻/⁻* (dark blue) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 (1 hour; MOI 10 - 50) followed by gentamicin treatment to remove extracellular bacteria. (A) The uptake of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. All experiments were performed at least twice with ≥ 3 technical repeats. Data are pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

3.2.8 Pyroptosis evasion by *Salmonella* Typhimurium results in an increased intracellular bacterial burden *in vitro*

Previous findings showed that *S*. Typhimurium mutant strains with defective SPI-1 T3SS or flagellin did not induced cell death of infected cells to the same extend like the WT strain (Jesenberger et al., 2000, Lightfield et al., 2008, Miao et al., 2006). To validate our previous results, we therefore infected WT BMDMs with *S*. Typhimurium SL1344 strains lacking flagellin or carrying mutations in structural parts of their T3SS encoded by SPI-1 to analyse their ability to induce macrophage cell death. BMDMs infected with a MOI of 10 – 50 of the *S*. Typhimurium mutant strain SL1344 *ΔinvA*, that does not possess an intact SPI-1 T3SS, did not undergo pyroptosis or pyroptosis-independent cell death (**Figure 3.14 A**). The non-flagellated *S*. Typhimurium strain SL1344 *ΔfliCΔfljB* did induce cell death in under 10 % (MOI 10) and 40% (MOI 50) of infected WT BMDMs, compared to 55 % and 80 % induced by the WT *S*. Typhimurium strain respectively. The reduced cell death rate observed was in line with significantly higher intracellular bacterial numbers of *S*. Typhimurium mutants recovered from infected BMDMs 2 and 6 hours post-infection (**Figure 3.14 B**). Notably, SL1344 *ΔinvA* infected BMDMs (MOI 10 and 50) did not induce noticeable cell death and harboured even more intracellular bacteria 2 and 6 hours post-infection than BMDMs infected with SL1344 *ΔfliCΔfljB*.

In summary, we verified the newly established *in vitro* assays by confirming and extending published findings on delayed cell death kinetics of pyroptosis deficient BMDMs after *S*. Typhimurium infection. We additionally validated previous results obtained by measuring the LDH release of dying cells 2, 6 and 24 hours post-infection (Doerflinger et al., 2020). This is in line with our *in vivo* observations that apoptosis compensates for the loss of pyroptosis and to ensure *S*. Typhimurium control. Additionally, we showed that *S*. Typhimurium bacteria lacking flagellin or an intact T3SS encoded by SPI-1 are not inducing pyroptosis to the extent of the

WT strain. Our findings indicate that *S*. Typhimurium survives and replicates in macrophages under conditions in which pyroptosis is absent or not induced. Importantly, our *in vivo* findings revealed that mice infected with flagellin or T3SS deficient *S*. Typhimurium strains control the infection (**Figure 3.3**), which indicates that additional mechanisms are in place *in vivo* to ensure infection control and clearance.

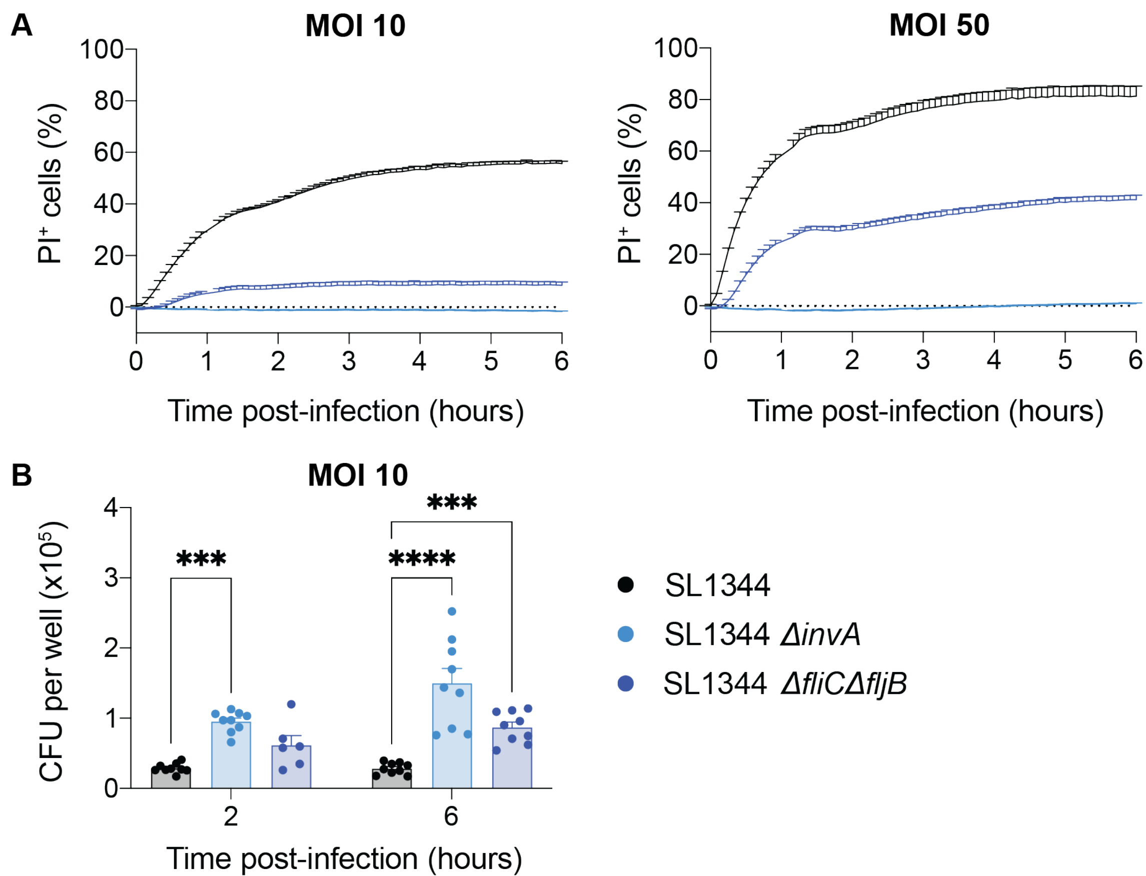


Figure 3.14: Pyroptosis evasion results in increased intracellular bacterial numbers.

(A and B) Wild-type (WT) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 (depicted in black), SL1344 *ΔinvA* (light blue) or SL1344 *ΔfliCΔfljB* (dark blue) infected (1 hour; MOI 10 - 50) followed by gentamicin treatment to remove extracellular bacteria. (A) The uptake of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. All experiments were performed at least twice with ≥ 3 technical repeats. Data are pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B) and calculated *p*-values are depicted (*** $p < 0.001$; **** $p < 0.0001$).

3.2.9 Combined absence of pyroptosis, necroptosis and extrinsic apoptosis renders macrophages resistant to *Salmonella* Typhimurium induced cell death *in vitro*

We next investigated if the delayed cell death induced by *S*. Typhimurium in pyroptosis deficient macrophages is orchestrated by caspase-8. Therefore, macrophages deficient for pyroptosis, necroptosis and extrinsic apoptosis were infected with MOI 50 of *S*. Typhimurium SL1344.

Casp1$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ BMDMs were resistant to *S*. Typhimurium SL1344 induced cell death, shown by the absence of PI incorporation after infection (**Figure 3.15 A**). The delayed apoptotic cell death observed in *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$ BMDMs was not initiated, reassuring that caspase-8 dependent apoptosis was induced in pyroptosis deficient macrophages. Besides that, *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ BMDMs harboured significantly higher numbers of intracellular bacteria 2 and 6 hours post-infection compared to WT BMDMs (**Figure 3.15 B**).

Taken together, pyroptosis, necroptosis and extrinsic apoptosis deficient macrophages are unable to undergo cell death to remove the intracellular replicative nice of *S*. Typhimurium and thereby restrict bacterial survival. Most likely, this accumulation of bacteria through unrestricted intracellular growth within macrophages results in severe systemic infection and death of infected mice observed *in vivo*.

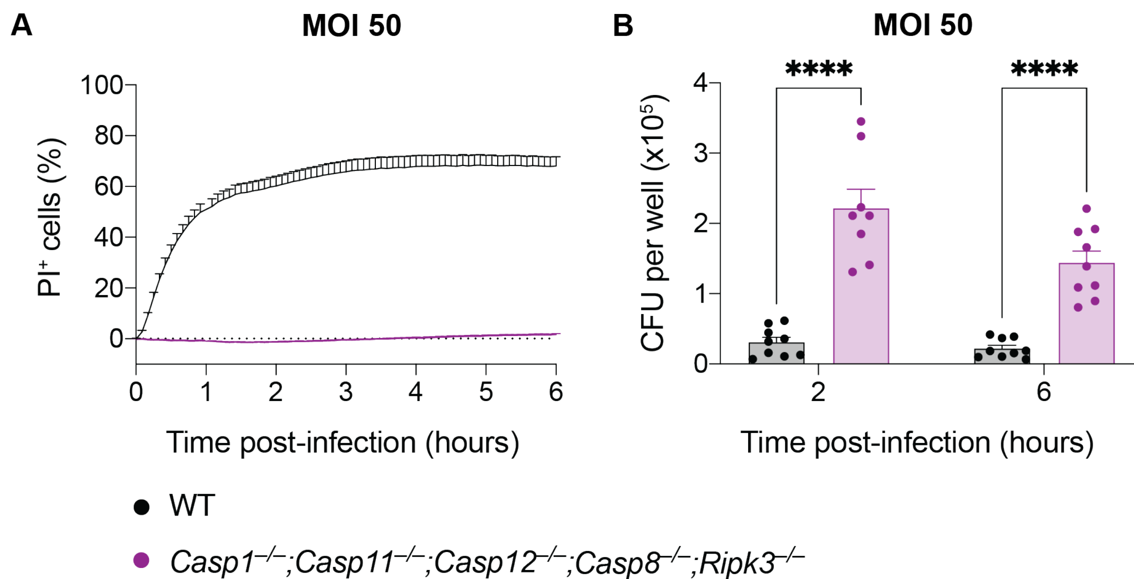


Figure 3.15: The combined loss of caspases-1, -11, -12, -8 and RIPK3 results in resistance to *Salmonella* Typhimurium induced cell death *in vitro*.
(A and B) Wild-type (WT; depicted in black) and *Casp1–/–;Casp11–/–;Casp12–/–;Casp8–/–;Ripk3–/–* (purple) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 (1 hour; MOI 50) followed by gentamicin treatment to remove extracellular bacteria. (A) The uptake of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. All experiments were performed at least three times with ≥ 2 technical repeats. Data are pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B) and calculated *p*-values are depicted (**** $p < 0.0001$).

3.3 Discussion

The importance of PCD as effective host immune defence against invading pathogens, the interconnectivity and redundancy of involved pathways and the essential role of macrophages in the control and clearance of intracellular infections has become increasingly evident in recent years.

Early publications investigating the host immune responses during infection with bacterial pathogens, including *Salmonella* spp. and *Shigella flexneri*, described the cell death observed in macrophage as apoptosis or necrosis initially (Moss et al., 1999, Navarre and Zychlinsky, 2000, Weinrauch and Zychlinsky, 1999). Macrophage death occurred within 30 minutes after infection with characteristic features of apoptosis, such as DNA fragmentation (Chen et al., 1996, Lindgren et al., 1996, Monack et al., 1996, Zychlinsky et al., 1992). Moreover, caspase-1 was considered to be an apoptotic caspase triggered by the *Salmonella* SPI-1 effector protein SipB (Hersh et al., 1999). However, these findings were published before it was known that additional types of PCD distinguishable from apoptosis exist. In the following years, pyroptosis and necroptosis were discovered and characterised as distinct cell death pathways, next to several additional form of lytic and non-lytic PCD. Thereafter, pyroptosis mediated by caspases-1 and -11 was described as the primary PCD pathway induced by infected phagocytes as defence mechanism against many intracellular bacterial pathogens (Jorgensen et al., 2017). Recent findings however imply the involvement of several PCD effectors and pathways in the control of *S*. Typhimurium infections (Jorgensen et al., 2017, Rauch et al., 2017, Van Opdenbosch et al., 2017).

Sophisticated knockout mouse and cell models, and high-resolution *in vitro* assays are needed to disentangle the complex network of PCD pathways involved in intracellular infection control, to analyse the roles of different cellular sensor and effector molecules therein, as well as address the double-edged role of macrophages as immune response regulator and replicative niche during intracellular infections. Multiple knockout mouse strains, deficient for different

combinations of caspases and RIPK3, have enabled us to study the relative importance of pyroptosis, necroptosis and apoptosis for control and clearance of *S*. Typhimurium infections.

In line with previous reports investigating the immune response to oral bacterial infections (Broz et al., 2010, Broz et al., 2012b, Knodler et al., 2014, Lara-Tejero et al., 2006, Man et al., 2014b, Raupach et al., 2006), we observed that pyroptosis deficient mice are more susceptible to *S*. Typhimurium using a systemic infection model of enteric fever. However, pyroptosis deficient mice cleared the infection similarly to WT mice, which implies that additional immune mechanisms contribute to the control of *S*. Typhimurium infections and argues against the sole involvement of pyroptotic PCD.

We revealed that caspase-12 is not required for PCD regulation during *S*. Typhimurium infection. Our findings confirm previous studies, which argued that caspase-12 harbours functions that are distinct from the described roles of caspases-1 and -11. For example, it was reported that caspase-12 does not process pro-IL-1β (Van de Craen et al., 1997). Besides that, no caspase-12 protein expression was observed in macrophages (Kalai et al., 2003). Furthermore, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ BMDMs treated with different cytotoxic agents induced cell death similar to WT cells (Salvamoser et al., 2019), which implies that caspase-12 is not involved in apoptotic or necroptotic cell death induction.

However, our findings revealed the involvement of apoptosis in the immune response to *S*. Typhimurium. The flexible induction of pyroptosis and apoptosis ensures *S*. Typhimurium control *in vivo*, as they effectively limit intracellular bacterial replication in macrophages as shown *in vitro*. In the combined absence of caspases-1 and -11 induced pyroptosis and caspase-8 mediated apoptosis, the control of *S*. Typhimurium infection is severely impaired and infected mice succumbed to infection. In contrast, necroptosis is not essential for the control of *S*. Typhimurium infections. This was confirmed by infection of necroptosis

deficient mice lacking the effector MLKL. The deletion of necroptosis alone did not substantially impair *S*. Typhimurium control *in vivo* (Doerflinger et al., 2020). Additionally, no phosphorylation of MLKL was observed in *S*. Typhimurium infected pyroptosis deficient cells *in vitro*, which indicates that necroptosis does not act as functional backup in the absence of caspases-1, -11 and -12 (Doerflinger et al., 2020). Our findings are in line with studies that investigated the role of PCD in immunity against the intracellular pathogen *Mycobacterium tuberculosis*. Pyroptosis deficient mice as well as caspase-8 mediated apoptosis deficient mice controlled *Mycobacterium tuberculosis* infection without major impairments (Stutz et al., 2021). Besides that, MLKL, RIPK3 and necroptosis were shown to be neither crucial for the immune response against *Mycobacterium tuberculosis* (Stutz et al., 2018a, Stutz et al., 2018b) nor *S*. Typhimurium (Robinson et al., 2012).

Our findings revealed that PCD induction of the hematopoietic cell compartment, including macrophages, is essential to restrict bacterial replication and control *S*. Typhimurium infections *in vivo*. We therefore infected macrophages to further resolve the mechanisms involved in the flexible induction of PCD pathways during intracellular infection. BMDMs have been established as a powerful tool and are widely used as preferred *in vitro* model to study macrophage functions (Assouvie et al., 2018, Glass and Natoli, 2016, Murray et al., 2014). BMDMs can be polarised and activated in multiple ways resulting in different activation states and phenotypes. Current existing BMDM generation protocols differ in cell culture media compositions and cell densities used for the differentiation process (Assouvie et al., 2018, de Brito Monteiro et al., 2020, Manzanero, 2012, Toda et al., 2021, Trouplin et al., 2013, Weischenfeldt and Porse, 2008, Zhang et al., 2008). It was shown that these differences influence cell surface marker expression, cytokine secretion and phagocytosis of generated BMDM cultures (de Brito Monteiro et al., 2020, Heap et al., 2021, Lee and Hu, 2013), which may impact the outcome of functional assays and reliability to compare findings obtained from different studies. Therefore, we characterised and validated our *in vitro* generated primary BMDMs. The F4/80 and CD11b expression of

differentiated BMDMs was comparable to published protocols (Assouvie et al., 2018, Toda et al., 2021, Ying et al., 2013). Additionally, generated BMDMs expressed the costimulatory molecules CD80 and CD86, which are typically found on APCs. Low basal expression levels of MHC-II and CD83 indicate that BMDMs were not highly activated following differentiation.

Common methods to analyse cell death, including LDH release assays, FACS analysis or microscopy, are not ideal to disentangle the complex cell death network and underlying kinetics due to several limitations. Conventional approaches are relatively time-consuming, resource demanding and comparably expensive. The disadvantages of LDH and TUNEL assays, which measure the release of LDH or detect fragmented DNA respectively, are relatively high background levels and the risk of false positive results for example. One caveat of FACS cell death analysis via Annexin V and PI staining is the fact that dead cells rapidly dissolve which makes the quantification of cell death difficult. However, the most important drawback of most assays is the fact that they provide only snapshots of cell death processes at distinct time points but are unable to resolve cell death kinetics continuously and in great detail as it unfolds, which is necessary to distinguish between different types of PCD. The assay established in this chapter can be performed in a 96-well format and therefore allows efficient high throughput analysis and continuous, real-time resolution of cell death kinetics. Using this to our advantage, we validated and extended previous findings by showing that inflammatory caspases-1, -11 and -12 deficient macrophages are unable to respond to infection through rapid pyroptosis induction (Jesenberger et al., 2000, Lara-Tejero et al., 2006, Lightfield et al., 2008, Mariathasan et al., 2004, Miao et al., 2006). More importantly however, we verified the observation that pyroptosis deficient cells undergo *S.* Typhimurium induced cell death with delayed kinetics instead (Jesenberger et al., 2000) and further characterised this phenomenon. Brightfield microscopy revealed that infected *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ macrophages undergo delayed cell death with apoptotic morphology, indicated by cellular fragmentation and formation of apoptotic bodies (Doerflinger et al., 2020). Immunoblotting confirmed that

pyroptosis deficient cells induce apoptosis by cleavage of initiator caspases-8 and -9 and subsequent effector caspases-3 and -7 activation as well as BID and poly-ADP ribose polymerase (PARP) (Doerflinger et al., 2020). However, GSDMD cleavage by activated caspase-8 or phosphorylation of MLKL was not observed. Additionally, we showed that *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ macrophages are resistant to *S.* Typhimurium induced killing and harboured large numbers of intracellular bacteria. Using confocal and lattice light-sheet imaging we confirmed that *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ macrophages contain large numbers of intracellular *S.* Typhimurium bacteria. Immunoblotting revealed that in the absence of caspase-8 effector caspases-3 and -7 were not activated (Doerflinger et al., 2020). In summary, we showed that caspase-8 mediated apoptosis is an effective mechanism to control *S*. Typhimurium infections if caspases-1 and -11 mediated pyroptosis is absent.

Detailed *in vitro* analysis using immortalised BMDMs deficient for different combinations of cell death initiators and effectors further revealed the complexity and interconnectivity of pyroptosis and apoptosis. The results indicate that caspases-1 and -8 can be used interchangeably between pathways. Caspase-1 is able to activate BID and the apoptotic caspases-3, -7 and -9 independently of caspase-8 (Doerflinger et al., 2020). This observation is consistent with recent reports implying that caspase-1 triggers apoptosis in GSDMD deficient cells by bypassing caspase-8 (Heilig et al., 2020, Tsuchiya et al., 2019). Caspase-1 was shown to cleave BID to release mitochondrial SMAC and trigger apoptosis via caspases-9 and -3 in poly(deoxyadenylic-deoxythymidylic) transfected BMDMs (Heilig et al., 2020). Similar results were obtained following *S*. Typhimurium infection of BMDMs (Tsuchiya et al., 2019). Additionally, this study indicates that caspase-3 cleaves gasdermin E thereby inducing secondary lytic cell death.

Interestingly, our results imply that caspases-1 and -8 harbour cell death executioner functions in the absence of all known downstream effectors of pyroptosis and apoptosis. Caspase-1 acts then upstream of and requires

caspase-8 to induce cell death. Furthermore, the findings indicate that caspase-11 can partially compensate for the loss of caspases-1 and -8 to ensure GSDMD-mediated cell death of infected macrophages (Doerflinger et al., 2020). This compensatory role of caspase-11 was previously investigated by other studies showing that caspase-11-dependent cell death contributes to macrophage death during *S*. Typhimurium infection (Broz et al., 2012b, Ng and Monack, 2013).

Besides that, we verified that *S*. Typhimurium mutant strains lacking flagellin or SPI-1 T3SS do not induce pyroptotic or apoptotic cell death *in vitro* to the same extend than WT *S*. Typhimurium as previously suggested by other studies (Jesenberger et al., 2000, Lightfield et al., 2008, Miao et al., 2006). This indicates that apoptosis induction in pyroptosis deficient macrophages *in vitro* is mediated by caspase-8 recruitment and activation via inflammasomes like previously implied by several studies (Man et al., 2014b, Man et al., 2013). Intracellular T3SS and flagellin deficient *S*. Typhimurium bacteria are not detected by the cellular sensor NLRC4 and therefore do not induce inflammasome assembly and intrinsic apoptosis in the absence of pyroptosis. Interestingly, we showed that T3SS and flagellin deficient *S*. Typhimurium strains are controlled without major impairments *in vivo*. Therefore, further experiments are required to characterise additional mechanisms of apoptosis induction that ensure the detection and clearance of mentioned *S*. Typhimurium mutant strains *in vivo*.

Taken together, our findings provide convincing evidence that pyroptosis and apoptosis are highly interconnected and induced with a remarkable level of redundancy following *S*. Typhimurium infections to remove intracellular bacteria residing within macrophages. The extensive crosstalk between pyroptosis, necroptosis and apoptosis following infection was also reported by other studies, which infected macrophages with *Yersinia pseudotuberculosis* (Malireddi et al., 2020) or influenza A virus, vesicular stomatitis virus, *Listeria monocytogenes* in addition to *S.* Typhimurium (Christgen et al., 2020). BMDMs deficient for caspases-1 and -11, GSDMD, GSDMD and MLKL, RIPK3 or RIPK3 and

caspase-8 induced PCD following infection with *S.* Typhimurium SL1344. The deletion of pyroptosis resulted in a reduced overall cell death rate, which reflects our observations. In contrast, combined absence of caspases-1, -11 and -8 together with RIPK3 resulted in resistance to *Salmonella* induced PCD. Furthermore, findings indicate that necroptosis is not crucial in the host response to *S.* Typhimurium infection. These results were in line with our findings presented in this chapter. Additionally, similar observations were obtained from infections of BMDMs with *Yersinia pseudotuberculosis*, *Listeria monocytogenes* or vesicular stomatitis virus. BMDMs underwent PCD following infection with both pathogens even if one or a combination of two different PCD pathways were absent. Surprisingly, a small fraction of *Listeria monocytogenes* and vesicular stomatitis virus infected BMDMs died even if pyroptosis, necroptosis and caspase-8 mediated apoptosis was deleted. This indicates that additional pathways, such as intrinsic apoptosis, are triggered by *Listeria monocytogenes* and vesicular stomatitis virus. In contrast, PCD induced following infections with influenza A virus seemed to depend on caspase-8.

The *in vitro* approaches established and refined in this chapter allow the analysis of cellular and molecular requirements for PCD induction, study cell death kinetics and help to understand the relative importance of different PCD pathways for the control and clearance of intracellular infections. Pathogenic factors important for triggering PCD can be determined by infecting cells with bacterial mutant strains that lack specific characteristics. On the other hand, host cell sensors and effectors essential for pathogen recognition and cell death regulation can be studied with cells deficient for distinct factors. Furthermore, the efficiency and effects of cell death inducing drugs can be analysed. Using cell co-cultures in these assays will allow to determine the cytotoxic abilities of cells and which cellular effectors these processes are mediated by.

Due to the observed complexity and interconnectivity of the PCD network it is most likely that additional so far unexplored links between pyroptosis and apoptosis exist. The established *in vitro* assays will be used in the following

chapters, together with sophisticated multiple knockout *in vivo* models, to further disentangle the interconnectivity, flexibility and regulation of pyroptosis and apoptosis that ensures intracellular infection control and clearance. This aims to reveal additional cellular and molecular requirements important for induction of PCD during *S*. Typhimurium infections.

Chapter 4:

The role of caspase-2 during *Salmonella* Typhimurium infection

4.1 Background

The findings presented and discussed in the previous chapter revealed that the flexible induction of pyroptosis and apoptosis following *S.* Typhimurium infection and the interchangeable use of caspases-1 and -8 between PCD pathways ensures infection control. However, the cellular and molecular requirements of apoptosis induction during *S.* Typhimurium infection are not completely understood. Due to the emerging interconnectivity and redundancy within the PCD network it is most likely that additionally roles of known cell death effectors as well as so far unidentified mediators contribute to immune responses against intracellular infections.

One additional PCD member that might be involved in the control of intracellular infections is caspase-2, which has been described as potential link between pyroptosis and apoptosis (Jesenberger et al., 2000). Caspase-2 was one of the first identified member of the caspase family and is the most evolutionarily conserved caspase across species (Lamkanfi et al., 2002). However, the function of caspase-2 remained relatively poorly understood for a long time (Fava et al., 2012, Kumar, 2007). Only a limited number of caspase-2 substrates are known (Brown-Suedel and Bouchier-Hayes, 2020) and their impact on cell death is still not fully investigated yet. It has been difficult to correctly place caspase-2 in the PCD cascades and determine its role in regulating apoptotic as well as non-apoptotic processes (Bouchier-Hayes and Green, 2012). Therefore, caspase-2 was referred to as orphan of the caspase family for many years (Krumschnabel et al., 2009, Vakifahmetoglu-Norberg and Zhivotovsky, 2010).

Caspase-2, now considered an apoptotic initiator caspase (Bouchier-Hayes and Green, 2012), is activated by proximity-induced dimerization (Bouchier-Hayes et al., 2009) facilitated by the molecular complex called p53-induced protein with a death domain (PIDD)osome (Tinel and Tschopp, 2004). Unlike other initiator caspases, caspase-2 does not activate downstream executioner caspases by proteolytic cleavage (Boatright and Salvesen, 2003) despite sharing overlapping

substrate specificity with them (Talanian et al., 1997, Thornberry et al., 1997, Wejda et al., 2012). Instead, caspase-2 induces intrinsic apoptosis through the mitochondrial pathway by proteolytic cleavage of the BH3-only domain protein BID (Bonzon et al., 2006, Guo et al., 2002).

Caspase-2 has been linked to the host response against intracellular infections, such as *Brucella*, *Staphylococcus aureus* and *Salmonella* (Bronner et al., 2013, Chen et al., 2011, Chen and He, 2009, Imre et al., 2012, Jesenberger et al., 2000), as well as DNA damage, endoplasmic reticulum stress and mitosis (Dawar et al., 2017, Fava et al., 2017). Previous findings indicated a role for caspase-2 in controlling PCD during *S.* Typhimurium infection of macrophages *in vitro* (Jesenberger et al., 2000). Jesenberger and colleagues reported that caspase-2 is activated simultaneously with, but independently of, caspase-1 following *S.* Typhimurium infection. Furthermore, the authors of that study described that caspase-1 activation as well as pyroptosis and apoptosis induction partially caspase-2 dependent. These *in vitro* results suggest caspase-2 as potential master regulator of pyroptosis and apoptosis orchestrating the flexible use PCD pathways during *S.* Typhimurium infections.

However, the role of caspase-2 in PCD regulation and *S.* Typhimurium control has not been investigated in complex *in vivo* settings yet. Additionally, the above mentioned studies do not take into account that different PCD pathways are tightly interconnected and regulated by a remarkable level of redundancy, whereby caspases can operate in multiple pathways and thus substitute for the absence of other caspases (Bedoui et al., 2020). Therefore, we hypothesised that so far unidentified roles for caspase-2 could be uncovered under conditions where all key caspases required for the host response to *S.* Typhimurium infection are absent. The aims of this chapter are to confirm and extend the current knowledge about the importance of caspase-2 for PCD induction *in vitro* using the assays established in the previous chapter and to investigate the relative contributions of caspase-2 to host defence against *S.* Typhimurium infections *in vivo* using novel knockout mouse models.

4.2 Results

4.2.1 Caspase-2 is not essential for programmed cell death induction following *Salmonella* Typhimurium infection *in vitro*

To investigate any primary roles of caspase-2 in PCD induction during *S.* Typhimurium infection, possibly by acting as link between pyroptosis and apoptosis, we compared the infection control of WT and caspase-2 deficient mice and BMDMs. Furthermore, we generated mice and BMDMs lacking caspase-2 additionally to caspases-1, -11, -12, -8 and RIPK3 to analyse potential compensatory roles of caspase-2 during intracellular infection.

We first examined how BMDMs respond to infection with MOI 50 of the *S*. Typhimurium strain SL1344. Up to 70 % of WT BMDMs were killed within 2 hours of infection as determined by the cellular incorporation of PI (**Figure 4.1 A**). *Casp2*$^{-/-}$ BMDMs showed a slightly reduced rate of cell death compared to WT BMDMs. We performed a LDH release assay as a different measurement of cell death. Measured cell death kinetics and rates of infected WT BMDMs were comparable between assays. However, the observed minor differences in the PI cell death kinetics assay between WT and *Casp2*$^{-/-}$ BMDMs were not evident in the LDH release assay (**Figure 4.1 B**). These findings were in line with comparable intracellular bacterial titers of *Casp2*$^{-/-}$ BMDMs and WT BMDMs 2 and 6 hours post-infection (**Figure 4.1 C**). Overall, the results suggest no critical role for caspase-2 in *S.* Typhimurium induced killing of macrophages *in vitro*.

As previously shown (**Figure 3.15**), *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ BMDMs were resistant to cell death upon SL1344 infection und harboured significantly more intracellular bacteria compared to WT BMDMs (**Figure 4.1 A and B**). Similar but not greater resistance was seen in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ BMDMs that additionally lacked caspase-2, indicating neither a potential pro- nor anti-

apoptotic role for caspase-2 during *S.* Typhimurium infection *in vitro*. These findings were confirmed as no differences in the intracellular bacterial numbers could be ascribed to the absence of caspase-2 in combination with caspases-1, -11, -12, -8 and RIPK3 at 2 and 6 hours post-infection (**Figure 4.1 C**).

These observations extend on previously published results which revealed that caspase-2 was required for early cell death induction by *S.* Typhimurium (Jesenberger et al., 2000). However, overall findings obtained from diverse *in vitro* assays indicate that caspase-2 does not play a substantial primary or compensatory role in *S.* Typhimurium induced cell death of BMDMs and hence the associated control of *S.* Typhimurium replication.

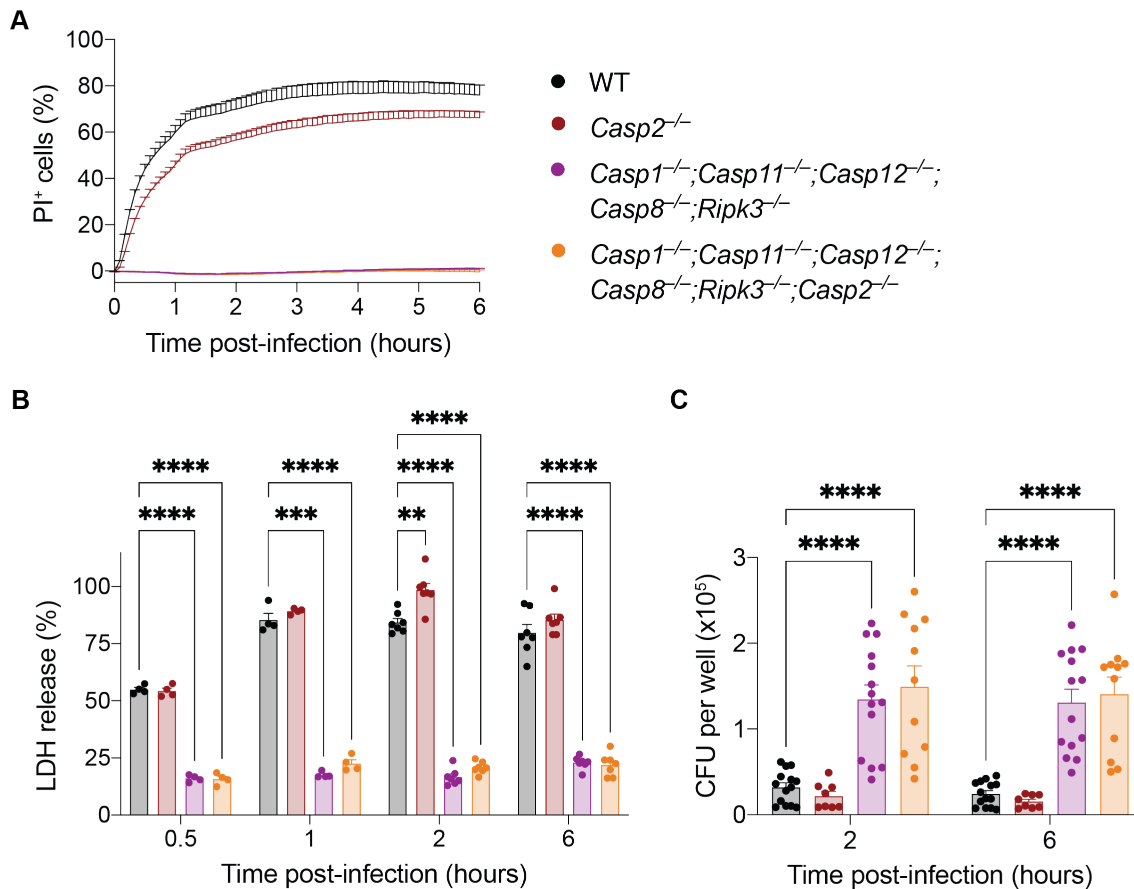


Figure 4.1: Caspase-2 does not play a critical role during programmed cell death induction by *Salmonella* Typhimurium *in vitro*.

Wild-type (WT; depicted in black), *Casp2–/–* (red), *Casp1–/–;Casp11–/–;Casp12–/–;Casp8–/–;Ripk3–/–* (purple) and *Casp1–/–;Casp11–/–;Casp12–/–;Casp8–/–;Ripk3–/–;Casp2–/–* (orange) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 (1 hour; MOI 25-50) followed by gentamicin treatment to remove extracellular bacteria. (A) The incorporation of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection (MOI 50). (B) The lactate dehydrogenase (LDH) release was analysed as an indicator for cell death at the indicated time points post-infection (MOI 50). (C) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection (MOI 25). All experiments were performed one to three times with ≥ 3 technical repeats. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B, C) and calculated *p*-values are depicted (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

4.2.2 Caspase-2 is not important for control and clearance of *Salmonella* Typhimurium infection *in vivo*

The full redundancy of the cell death processes that ensure host protection during intracellular infections becomes obvious under *in vivo* conditions. Given that the role of caspase-2 in bacterial control and clearance has not yet been determined *in vivo*, we infected mice with 200 CFU of the growth attenuated *S.* Typhimurium strain BRD509, which results in a mild systemic infection that can be controlled in WT mice (Kupz et al., 2014).

Focusing our analysis on the peak of infection, we found that bacterial titers in the liver and spleen 3 weeks post-infection were comparable in WT and *Casp2*$^{-/-}$ mice (**Figure 4.2 A**), suggesting no critical role for caspase-2 in *S*. Typhimurium control *in vivo*. As shown previously (**Figure 3.6 A and 3.8**), such control was compromised in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice resulting in significantly elevated bacterial titers (**Figure 4.2 A**). The additional absence of caspase-2 did not cause a marked difference with only a minor drop in bacterial titers in the *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ mice compared to *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice.

To investigate the impact of systemic *S*. Typhimurium infection on the wellbeing of mice, we additionally monitored indicators such as changes in body weight, physical appearance and behaviour. Pronounced loss of body weight, due to decreased water and food intake, and reduced responsiveness indicate severe infection. No changes in the behaviour of infected mice were detected during the first three weeks post-infection. Furthermore, the absence of caspase-2 alone or in combination with caspases-1, -11, -12, -8 and RIPK3 did not result in weight loss over the analysed time period compared to WT or *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice respectively (**Figure 4.2 B**).

Liver and spleen are major replication sites of *S*. Typhimurium *in vivo* resulting in symptoms, such as hepatomegaly and splenomegaly, which advance with disease severity. This increase in organ size could potentially compensate for any body weight loss during infection. We therefore also analysed the weights of liver and spleen at the peak of infection. *Casp2*$^{–/–}$ mice did not show increased organ sizes compared to WT mice 3 weeks post-infection. The liver and spleen weights of infected *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$*;Casp8*$^{–/–}$*;Ripk3*$^{–/–}$ and *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$*;Casp8*$^{–/–}$*;Ripk3*$^{–/–}$*;Casp2*$^{–/–}$ mice were increased to a similar extend compared to WT mice (**Figure 4.2 C**). The observed severely enhanced hepatomegaly and splenomegaly was in line with significantly increased bacterial titers and confirmed the impaired infection control of *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$*;Casp8*$^{–/–}$*;Ripk3*$^{–/–}$ and *Casp1*$^{–/–}$*;Casp11*$^{–/–}$*;Casp12*$^{–/–}$*;Casp8*$^{–/–}$*;Ripk3*$^{–/–}$*;Casp2*$^{–/–}$ mice. However, the additional deletion of caspase-2 besides caspases-1, -11, -12, -8 and RIPK3 did not further increase organ weights.

Taken together, these results indicate that caspase-2 does not play a substantial role in *S*. Typhimurium control within the first 3 weeks post-infection *in vivo*, even under conditions that obviate potential compensatory roles by other caspases.

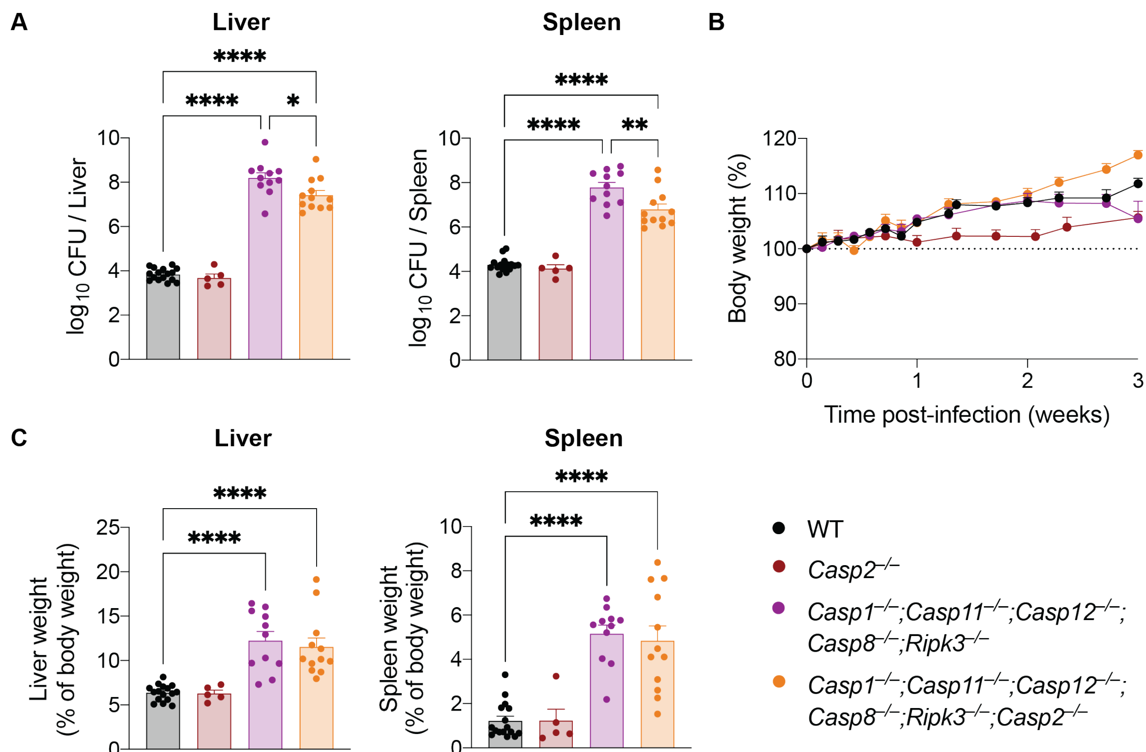


Figure 4.2: Caspase-2 does not play an important role in *Salmonella* Typhimurium control *in vivo*.

Wild-type (WT; depicted in black), *Casp2*$^{-/-}$ (red), *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ (purple) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ (orange) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed two to three times with each experimental group including $n \geq 2$. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by one-way ANOVA (A, C) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

WT mice clear systemic infections with the growth attenuated *S.* Typhimurium strain BRD509 within 12 weeks. To analyse the impact of caspase-2 deficiency on *S.* Typhimurium clearance, we next focussed on time points past 3 weeks post-infection. Therefore, we infected WT and *Casp2*$^{-/-}$ mice with 200 CFU of BRD509 and analysed their bacterial titers in liver and spleen 7 and 12 weeks post-infection. *Casp2*$^{-/-}$ mice showed no impairments in *S.* Typhimurium clearance indicated by declining bacterial titers and cleared the infection like WT mice within 12 weeks (**Figure 4.3 A**). In line with this, *Casp2*$^{-/-}$ mice constantly gained weight and did not show any signs of severe disease similar to WT mice (**Figure 4.3 B**). Furthermore, the liver and spleen weights of WT and *Casp2*$^{-/-}$ mice decreased back to baseline levels between 7 and 12 weeks post-infection (**Figure 4.3 C**) indicating restriction of bacterial growth and infection clearance.

Overall, these results imply that caspase-2 does not play an essential primary role in the clearance of *S.* Typhimurium infection *in vivo*.

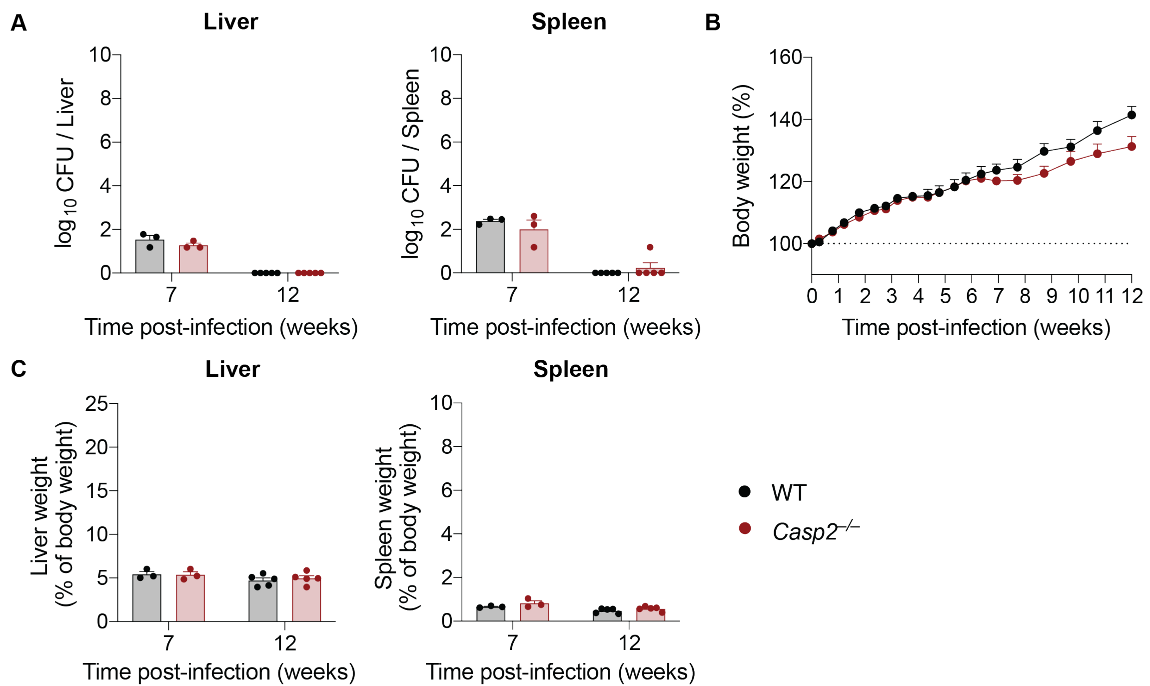


Figure 4.3: Caspase-2 does not play an essential primary role in *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *Casp2*$^{-/-}$ (red) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen were determined 7 and 12 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed once with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. No statistically significant differences were determined by two-way ANOVA (A, C).

To analyse the potential role of caspase-2 during the clearance of *S.* Typhimurium infections without the compensatory effects of other caspases, we next infected $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-};Casp2^{-/-}$ mice and compared their infection progression past 3 weeks post-infection to $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ mice. As shown previously (**Figure 3.7**), *S.* Typhimurium clearance was severely compromised in $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ mice, resulting in the death of infected mice (**Figure 4.4 A**). $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ mice succumbed to infection between day 22 and 30 post-infection. The additional absence of caspase-2 did result in a slight delay in the survival (**Figure 4.4 A**). $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-};Casp2^{-/-}$ succumbed to infection between day 26 to 34 post-infection, around 4 days later than mice competent in caspase-2. Additionally, $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ mice developed signs of severe disease, such as loss of body weight, overall physical appearance and reduced responsiveness, slightly earlier than $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-};Casp2^{-/-}$ mice (**Figure 4.4 B**). In contrast to that, WT mice did not succumb to infection and no body weight loss was detected (**Figure 4.4 A and B**). Next, we analysed the bacterial titers in liver and spleen at the time of sacrifice and compared them to infected WT mice. Bacterial titers of $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ and $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-};Casp2^{-/-}$ mice were elevated to the same extend (**Figure 4.4 C**) and even higher than 3 weeks post-infection (**Figure 4.2 A**). Additionally, we investigated bacterial numbers in the blood of infected mice to determine if uncontrolled bacterial replication in liver and spleen results in bacteraemia and the further spread of *S.* Typhimurium. In contrast to WT mice, we detected *S.* Typhimurium in comparable high numbers in the blood of $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ and $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-};Casp2^{-/-}$ mice indicating extensive bacterial replication and systemic dissemination via the bloodstream (**Figure 4.4 C**). Liver and spleen weights of $Casp1^{-/-};Casp11^{-/-};Casp12^{-/-};Casp8^{-/-};Ripk3^{-/-}$ and

Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$;Casp2$^{-/-}$ mice were significantly increased at the time of sacrifice compared to WT mice (**Figure 4.4 D**) and even higher than 3 weeks post-infection (**Figure 4.2 C**). The additional absence of caspase-2 did result in slightly higher spleen weights, while liver weights were comparable (**Figure 4.4 D**). Observed hepatomegaly and splenomegaly in *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$* and *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$;Casp8$^{-/-}$;Ripk3$^{-/-}$;Casp2$^{-/-}$* mice progressed with infection duration, which highlights the disease severity and impaired *S.* Typhimurium control of those mice.

These results indicate that caspase-2 does not play a redundant role in the clearance of *S.* Typhimurium infections *in vivo* under conditions that prevent any potential compensatory roles by other caspases.

In summary, our findings suggest that caspase-2 is not required for sufficient activation of caspases, such as caspases-1 and -8, important for *S.* Typhimurium control and clearance. Furthermore, the results indicate that caspase-2 does not play an important primary or compensatory role in the induction of pyroptosis and apoptosis after *S.* Typhimurium infection.

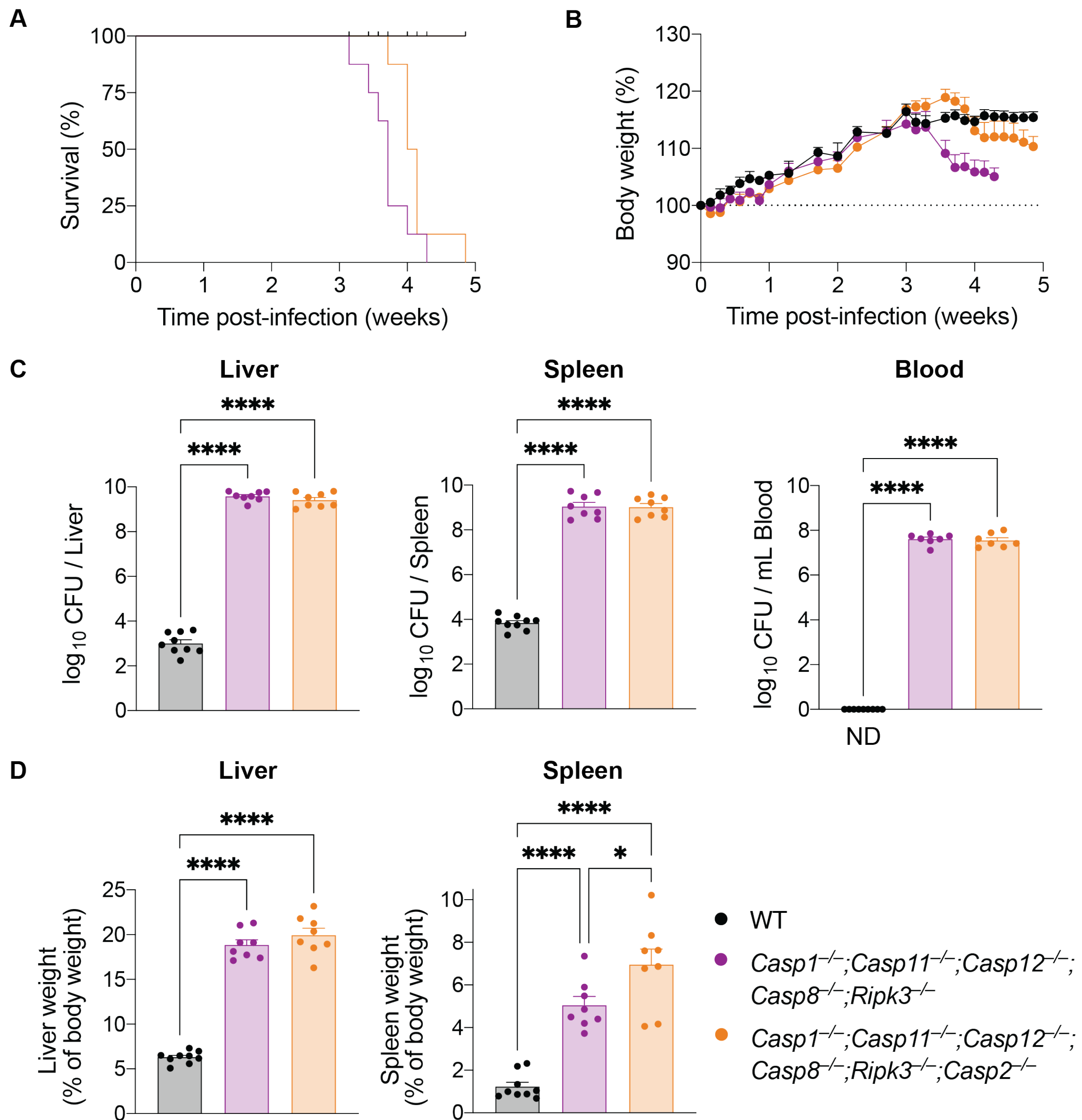


Figure 4.4: Caspase-2 does not play a compensatory role in *Salmonella* Typhimurium clearance *in vivo*.

Wild-type (WT; depicted in black), *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ (purple) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ (orange) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) The survival of infected mice was analysed based on body condition, physical appearance and behaviour. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Bacterial titers in the liver, spleen and blood were determined at the time of sacrifice. ND = not detected. (D) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of sacrifice. All experiments were performed twice with each

experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by log rank (Mantel cox) test with the Bonferroni-corrected threshold of 0.0167 (A) or one-way ANOVA (C, D) test and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

4.3 Discussion

Our findings using sophisticated knockout cell and mouse models reveal that the absence of caspase-2 causes no major impairment of *S.* Typhimurium control and clearance *in vitro* and *in vivo* and therefore argue against a significant role for caspase-2 operating as a fail-safe mechanism in the complex PCD network (Bedoui et al., 2020).

The mechanism by which the absence of caspase-2 reduces (albeit to a minor extent) the increase in bacterial burden caused by the loss of caspases-1, -11, -12, -8 and RIPK3 is not known. It may relate to its proposed roles in cell survival and cell division. In the complex situation of an *in vivo* *S.* Typhimurium infection, caspase-2 could act as a pro-survival factor for activated macrophages in the absence of other caspases. Its absence would thus lead to a decrease in the number of macrophages that can be infected, which would reduce the replicative niche for the bacteria.

The lack of a clear phenotype of the caspase-2 knockout mice following *S.* Typhimurium infection together with other studies demonstrating a limited role of caspase-2 in pathogen-induced cell death, raises the question whether caspase-2 plays any role in bacteria-induced cell death. There are reports that caspase-2 is of significant importance in infections with *Brucella abortus* and *Brucella suis* of macrophages (Bronner et al., 2013, Chen et al., 2011, Chen and He, 2009), where rough attenuated variants appear to induce a so-called hybrid form of cell death that combines features of both apoptosis and pyroptosis (Bronner et al., 2013) and is accompanied by endoplasmic reticulum stress leading to mitochondrial damage, inflammasome activation and pro-inflammatory cytokine release (Bronner et al., 2015). However, other studies found no evidence for a role of caspase-2 following rough attenuated *Brucella* infection (Tian et al., 2020). It is noteworthy that unattenuated smooth *Brucella* WT strains prevent macrophage death to establish replication and chronic infection (Chen et al., 2011, Chen and He, 2009). With the suggestion that only attenuated rough

Brucella variants can induce some cell death after inoculation with relatively high MOI (Bronner et al., 2015, Bronner et al., 2013, Chen et al., 2011, Chen and He, 2009), it is possible that there might be specific conditions under which caspase-2 can contribute to bacteria-induced cell death. However, our findings argue against a major role for caspase-2 that can be generalised across many intracellular bacteria. Caspase-2 does not orchestrate the flexible induction of pyroptosis and apoptosis in *S.* Typhimurium infected cell. Therefore, additional cellular and molecular requirements of apoptosis induction must be in place that ensure the control and clearance of *S.* Typhimurium infections.

Chapter 5:

The role of CD4$^+$ T cells in the initiation of extrinsic apoptosis during *Salmonella* Typhimurium infection

5.1 Background

The results presented in the previous chapters revealed that pyroptosis and apoptosis are essential for *S.* Typhimurium control. Both cell death pathways are highly interconnected and flexibly induced by infected host cells to limit intracellular bacterial replication and enhance elimination. Furthermore, we showed that caspase-2 does not act as link between pyroptosis and apoptosis and is not essential for immunity against *S.* Typhimurium infection. We still do not fully understand how apoptosis is induced in situations in which *S.* Typhimurium directly evades inflammasome detection and thereby avoids pyroptosis induction. Several publications implied that caspase-8 mediated cell death can be induced via inflammasomes activated by cytosolic *S.* Typhimurium PAMPs (Man et al., 2014b, Man et al., 2013). However, intracellular *S.* Typhimurium bacteria downregulate many PAMPs, including the SPI-1 encoded T3SS and flagellin, and modify their surface to avoid detection and inflammasome assembly after cell invasion (Cirillo et al., 1998, Cummings et al., 2006, Galan, 1999, Galan and Collmer, 1999, Galan and Curtiss, 1989, Galan and Zhou, 2000, Hensel et al., 1998, Lai et al., 2013, Miao et al., 2010a, Mills et al., 1995, Ochman et al., 1996, Shea et al., 1996). We showed that mice infected with flagellin or SPI-1 T3SS deficient *S.* Typhimurium strains control the infection *in vivo*, despite the observation that infected BMDMs did not undergo cell death *in vitro*. Therefore, we conclude that extrinsic apoptosis, induced by additional cellular and molecular mechanisms within the complex *in vivo* system, efficiently restricts *S.* Typhimurium infections even if inflammasome driven pyroptosis is absent. Extrinsic apoptosis is induced by ligation of TNF superfamily members, including TNF-α, FasL and TRAIL, to their respective receptors, TNFR1, Fas and TRAIL-R, on the surface of cells and subsequent activation of caspase-8.

We speculate that $CD4^+$ T cells and IFN-γ could play a crucial function in orchestrating the flexible use of extrinsic apoptosis because of their previously observed importance in immunity against *S.* Typhimurium.

Published findings showed that mice lacking functional $CD4^+$ T cells are unable to clear the growth attenuated *S.* Typhimurium and develop a chronic infection, which indicates that $CD4^+$ T cells are important for bacterial clearance (Hess et al., 1996, Kupz et al., 2014). In contrast to that, mice lacking $CD8^+$ T cells or B cells clear primary infections with attenuated *S.* Typhimurium like WT mice (Hess et al., 1996, Kupz et al., 2014). Further studies investigated the importance of $CD4^+$ T cells for the control of *S.* Typhimurium using a chronic infection model. 129X1/SvJ mice, which express functional Nramp1, develop a chronic infection when infected with the WT *S.* Typhimurium strain SL1344 (Goldberg et al., 2018, Loomis et al., 2014, Monack et al., 2004). The results of this study indicate that $CD4^+$ T cells, but not $CD8^+$ T cells, are required to restrict persistent *S.* Typhimurium infections (Goldberg et al., 2018, Johanns et al., 2010). This observation was also evident in infections with *Mycobacterium tuberculosis*. Tuberculosis in mice was enhanced in mice lacking functional $CD4^+$ T cells, while the absence of the $CD8^+$ T cells response had no impact (Mogues et al., 2001).

Th1 cells, a subset of $CD4^+$ T cells induced by *S.* Typhimurium infections (Goldberg et al., 2018, Monack et al., 2004), are one of the main sources of IFN-γ. The cytokine IFN-γ is a potent activator of innate immune cells, such as macrophages and neutrophils, and promotes phagocytosis, pro-inflammatory cytokine secretion and antimicrobial effector functions, such as ROS production (Reljic, 2007, Schroder et al., 2004). It was shown that IFN-γ and IFN-γ receptor deficient mice are unable to limit the replication of attenuated *S.* Typhimurium, therefore fail to induce the plateau phase of bacterial titers early on and subsequently succumb to infection (Hess et al., 1996, Kupz et al., 2014). This increased disease severity observed in *IFN-$\gamma^{-/-}$* mice highlights that not only $CD4^+$ T cells but also other cells produce IFN-γ, which is required to control the infection. Previous studies analysed the contribution of individual lymphocyte subsets by measuring serum IFN-γ levels of different knockout mouse lines after infection with attenuated *S.* Typhimurium (Kupz et al., 2014). The findings revealed that $CD8^+$ T cells and NK cells contribute to the IFN-γ secretion and can compensate the absence of $CD4^+$ T cells or $CD4^+$ and $CD8^+$ T cells, respectively

(Kupz et al., 2014). However, despite the production of IFN-γ by other lymphocytes, *S.* Typhimurium BRD509 infections are not cleared if mice lack $CD4^+$ T cells (Kupz et al., 2013, Kupz et al., 2014). Together with our findings presented in chapter 3, which revealed that PCD deficient mice are unable to control and clear *S.* Typhimurium BRD509 infections despite the presence of IFN-γ, this implies that cell death induction regulated by $CD4^+$ T cell is critical for the control and clearance of *S.* Typhimurium *in vivo*.

This hypothesis is furthermore supported by reports that investigated the control of *S.* Typhimurium infections in 129X1/SvJ mice, which suggest bacteria persist in macrophages within granulomas (Goldberg et al., 2018, Monack et al., 2004, Pham et al., 2020). *S.* Typhimurium was detected in activated, iNOS expressing macrophages (Goldberg et al., 2018, Pham et al., 2020) protected by the induction of genes that break down NO produced by iNOS (Goldberg et al., 2018). The IFN-γ mediated activation of macrophages is essential to limit bacterial replication (Monack et al., 2004) but is not efficient enough to eliminate *S.* Typhimurium from the host resulting in chronic infection of mice. Another reason for the persistence of *S.* Typhimurium could be the finding that Th1 cells were excluded from granulomas (Goldberg et al., 2018).

All observations combined implicate, that mechanisms mediated by $CD4^+$ T cells, besides their well described helper functions, are essential for the clearance of intracellular bacteria. Interestingly, it was reported that IFN-γ promotes the upregulation of genes involved in antigen presentation and the regulation of PCD via the JAK/STAT/interferon regulatory factor 1 (IRF1) pathway (Reljic, 2007, Schroder et al., 2004). IFN-γ upregulates the genes involved in MHC-II antigen presentation (Chang and Flavell, 1995, Cresswell, 1994, Figueiredo et al., 1989, Kern et al., 1995, Lah et al., 1995, Mach et al., 1996, Wolf and Ploegh, 1995), as well as apoptotic caspases (Dai and Krantz, 1999, Fulda and Debatin, 2002, Ruiz-Ruiz et al., 2004, Tekautz et al., 2006) and death ligands and receptors, including Fas/FasL (Xu et al., 1998, Zheng et al., 2002) and TNFR (Tsujimoto et al., 1986). Besides that, $CD4^+$ T cells have been shown to directly kill target cells

by recognition of the peptide-MHC-II complex by $\alpha\beta$ TCRs (Cenerenti et al., 2022). One mechanism through which cytotoxic $CD4^+$ and $CD8^+$ T cells can induce cell death is the release of perforin and granzymes, which perforate the plasma membrane of target cells and subsequently induce the caspase cascade. This is often described as main pathway how cytotoxic $CD8^+$ T cells carry out their macrophage killing function (Stenger et al., 1997). However, cytotoxic T cells also express and secrete members of the TNF superfamily, such as TNF-α, FasL and TRAIL, which are potent initiators of apoptotic cell death (Nagata, 1997). FasL induced apoptosis was described as the major cytotoxic mechanism of $CD4^+$ T cells used to induce cell death of target cells (Kagi et al., 1994, Nagata and Golstein, 1995, Stalder et al., 1994), including activated macrophages (Ashany et al., 1995).

Strikingly, it was not investigated yet if the observed impaired *S.* Typhimurium control and clearance in IFN-γ and $CD4^+$ T cells deficient mice is in part due to diminished induction of PCD processes by the death receptor ligands TNF-α, FasL and TRAIL. We hypothesise that novel roles for cytotoxic $CD4^+$ T cells and IFN-γ in antibacterial immunity and initiation of extrinsic apoptosis could be uncovered under conditions in which compensatory host immune mechanisms for *S.* Typhimurium control are absent.

The aim of the following chapter is to unravel novel roles for $CD4^+$ T cells and IFN-γ in extrinsic apoptosis induction and their contribution to the flexible usage of different PCD pathways during the host immune response to *S.* Typhimurium infection. We infected knockout mouse models and performed *in vivo* depletions to analyse the importance of $CD4^+$ T cells and their effector molecules for the induction of PCD. To further analyse potential cytotoxic functions of $CD4^+$ T cells and the underlying effector mechanisms, we modified the PI incorporation assay established in chapter 3 to measure the cell death kinetics of BMDMs that were co-cultured with $CD4^+$ T cells *in vitro*.

5.2 Results

5.2.1 TNF-α is not critical for control of *Salmonella* Typhimurium infection

One important immunomodulatory effector molecule released by myeloid cells and lymphocytes during intracellular bacterial infections is the cytokine TNF-α. Infections with *S.* Typhimurium induces the production of TNF-α by macrophages and epithelial cells (Ciacci-Woolwine et al., 1998, Jung et al., 1995, Pietila et al., 2005). Previous reports indicated that TNF-α secretion restricts bacterial replication during chronic infection with *S.* Typhimurium (Mastroeni et al., 1995, Pham et al., 2020). The depletion of TNF-α resulted in altered granuloma morphology and increased bacterial titers in the liver and spleen of infected mice (Mastroeni et al., 1995, Pham et al., 2020). However, the *in vivo* mechanisms of TNF-α in immunity against *S.* Typhimurium are incompletely understood. One study implied that TNF-α signalling promotes macrophage polarisation towards a proinflammatory phenotype (Pham et al., 2020). The possible role of TNF-α in the induction of extrinsic apoptosis during *S.* Typhimurium as one potential mechanism to limit bacterial replication and control the infection has not been investigated yet.

We first infected TNF-α deficient mice with *S.* Typhimurium to analyse the importance of TNF-α within our *in vivo* infection model. $TNF^{-/-}$ mice infected with 200 CFU of the growth attenuated *S.* Typhimurium strain SL1344 *ΔaroA* had similar bacterial titers in liver and spleen compared to WT mice 3 weeks post-infection (**Figure 5.1 A**), which suggests that TNF-α is not critical for the control of *S.* Typhimurium *in vivo* if all PCD pathways can be induced. Both mouse strains likely induce pyroptosis to control intracellular bacterial replication. Our results seem to be in contrast with above discussed studies describing a prominent role for TNF-α. However, these findings were obtained using chronic infection models, in which intracellular *S.* Typhimurium bacteria most likely evade pyroptosis, for example by downregulation of inflammasome activating PAMPs. To analyse the

importance of TNF-α for infection control in the absence of pyroptosis, we therefore infected *TNF*$^{-/-}$ mice with the growth attenuated, flagellin deficient *S.* Typhimurium strain SL1344 *ΔaroAΔfliCΔfljB*. As previously shown (**Figure 3.14 A**), the flagellin deficient strain does not induce pyroptosis to the same extend as the flagellin expressing *S.* Typhimurium strain. Infections with SL1344 *ΔaroAΔfliCΔfljB* did not result in elevated bacterial numbers in WT mice, which are able to induce apoptosis instead of pyroptosis (**Figure 5.1 A**). In contrast, *TNF*$^{-/-}$ mice infected with flagellin deficient *S.* Typhimurium showed significantly higher bacterial titers in liver and spleen compared to WT mice infected with the same strain. Additionally, infection of *TNF*$^{-/-}$ mice with SL1344 *ΔaroAΔfliCΔfljB* resulted in a significantly higher bacterial burden than SL1344 *ΔaroA* infection, suggesting that TNF-α contributes to infection control during pyroptosis evasion. Infected mice did not display any physical signs of distress or significant loss of body weight (**Figure 5.1 B**). Compared to WT mice, *TNF*$^{-/-}$ mice showed increased hepato- and splenomegaly after *S.* Typhimurium infection (**Figure 5.1 C**). However, the infection with SL1344 *ΔaroAΔfliCΔfljB* did not increase the organ weights of WT and *TNF*$^{-/-}$ mice further compared to the flagellin competent strain SL1344 *ΔaroA*.

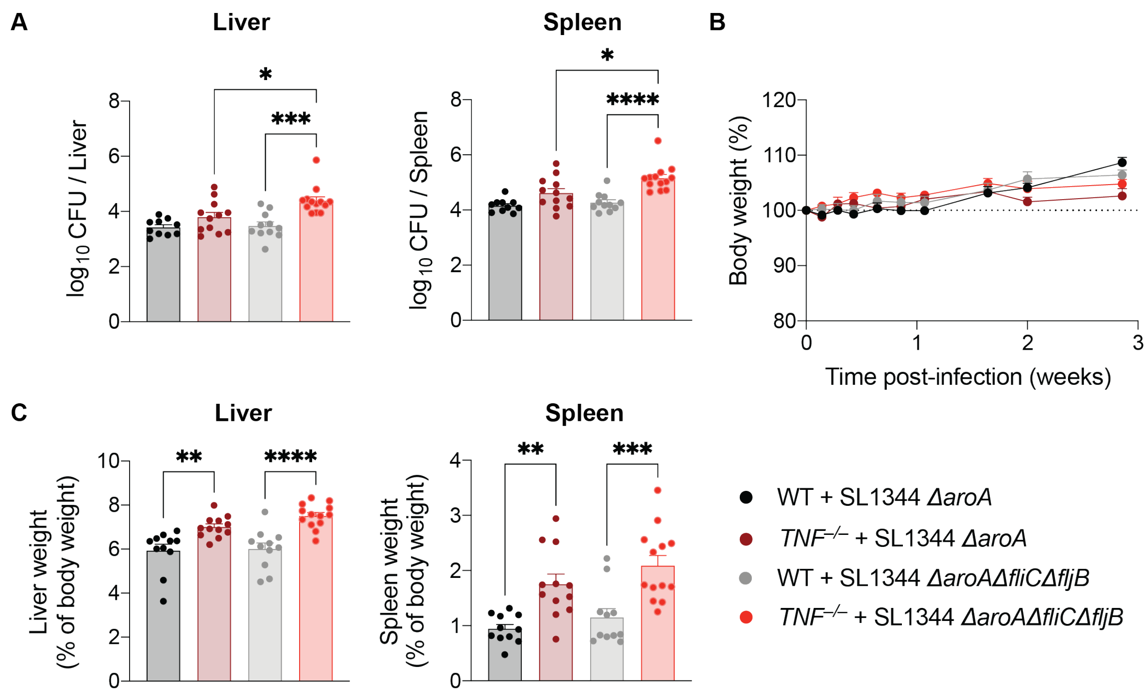


Figure 5.1: TNF-α plays a minor role in the control of pyroptosis evading *Salmonella* Typhimurium *in vivo*.

Wild-type (WT; depicted in black) and *TNF*$^{-/-}$ (red) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain SL1344 *ΔaroA* or the growth-attenuated, flagellin deficient mutant strain SL1344 *ΔaroAΔfliCΔfljB*. (A) Bacterial titers in the liver and spleen of WT and *TNF*$^{-/-}$ mice were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed three times with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by one-way ANOVA (A and C) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

We validated previous results, which indicate a surprisingly minor role for TNF-α in immunity against *S.* Typhimurium, using an independent experimental approach. To analyse the potential role of TNF-α in the initiation of apoptosis during intracellular infections, we infected WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice with 200 CFU of the growth attenuated *S.* Typhimurium strain BRD509 and inhibited TNF-α by injections of the TNF scavenger etanercept twice weekly. Etanercept is a TNF receptor fusion protein that binds to TNF and acts as decoy receptor thereby reducing the amount of available TNF (Wong et al., 2008). The bacterial titers in liver and spleen of infected mice were analysed 3 weeks post-infection, at the peak of infection in WT mice.

As previously shown (**Figure 3.1 A**), pyroptosis deficient mice have slightly elevated bacterial loads in liver and spleen compared to WT mice (**Figure 5.2 A**). The inhibition of TNF-α did not impact *S.* Typhimurium titers in the spleens of WT mice and resulted in a minor increase in the liver bacterial burden compared to the PBS control group. However, this increase in the liver *S.* Typhimurium titers between PBS and etanercept treated mice was not consistently observed. The bacterial numbers between groups were comparable in prior performed titration experiments, testing different concentrations of etanercept *in vivo* (data not shown). Interestingly, the inhibition of TNF-α by etanercept did only result in a small increase in the *S.* Typhimurium titers of pyroptosis deficient *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice. In line with this, mice did not display any physical signs of distress or loss of body weight (**Figure 5.2 B**). Furthermore, we did not observe any severe hepato- or splenomegaly induced by TNF-α blockade (**Figure 5.2 C**). No significant differences in liver and spleen weights within the etanercept and PBS groups or between compared WT mice was detected. However, inhibition of TNF-α led to a slight increase in the organ weights of infected *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice.

Interestingly, the bacterial titers of *TNF*$^{-/-}$ mice infected with SL1344 *ΔaroAΔfliCΔfljB* and TNF-α depleted *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice infected with *S.* Typhimurium BRD509 were not elevated to the same extend than previously observed in pyroptosis, necroptosis and apoptosis deficient *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice 3 weeks post-infection (**Figure 3.6 A and 3.8**). Collectively, these findings indicate that TNF-α plays a minor role in *S.* Typhimurium control and apoptosis induction *in vivo* and imply the involvement of additional cell death inducing effector molecules.

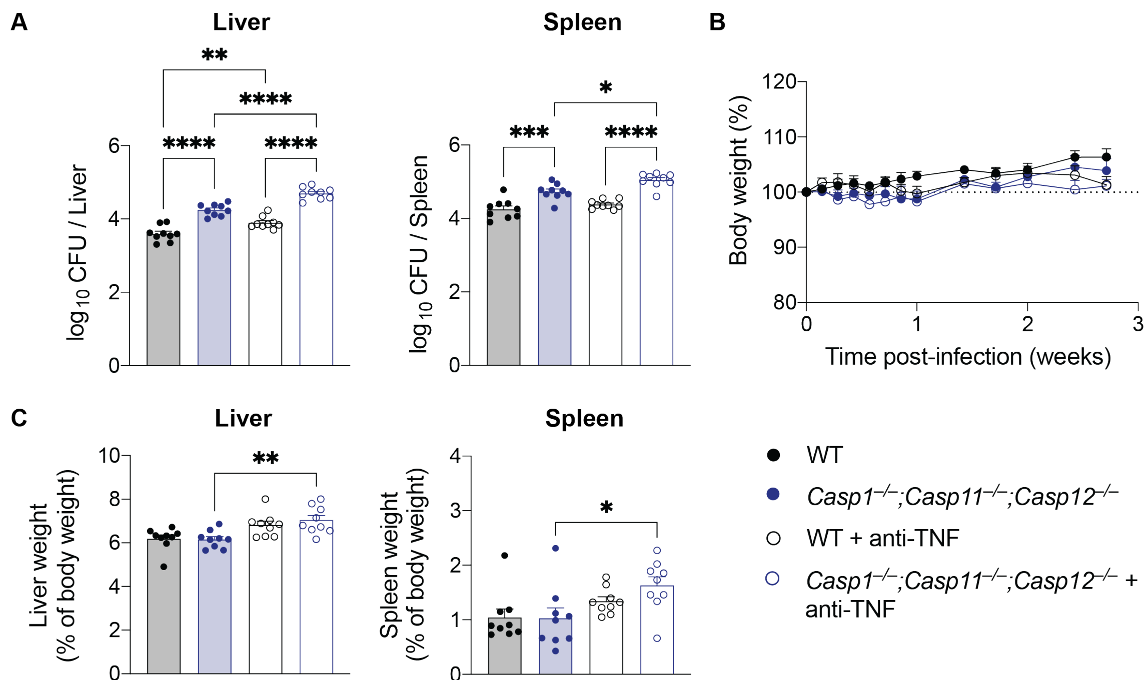


Figure 5.2: TNF-α plays a minor role for *Salmonella* Typhimurium control in the absence of pyroptosis *in vivo*.

Wild-type (WT; depicted in black) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ (blue) mice were injected intraperitoneally with etanercept (anti-TNF; open circles) or phosphate buffered saline (PBS; closed circles) twice weekly and infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen of WT and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed twice with each experimental group including $n \geq 4$. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by one-way ANOVA (A and C) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

To verify the interpretation of these findings, we next investigated the role of TNF-α for induction of PCD and *S*. Typhimurium control *in vitro*. WT and *TNF*$^{-/-}$ BMDMs were infected with MOI 10 of the *S*. Typhimurium strains SL1344 or the non-flagellated strain SL1344 *ΔfliCΔfljB* and cell death kinetics as well as intracellular bacterial burden measured. No major differences in cell death induction, indicated by the incorporation of PI, between WT and *TNF*$^{-/-}$ BMDMs were detected (**Figure 5.3 A**). The intracellular burden of infected BMDMs reflected these findings as comparable bacterial numbers were detected in WT and *TNF*$^{-/-}$ BMDMs (**Figure 5.3 B**). The non-flagellated *S*. Typhimurium strain SL1344 *ΔfliCΔfljB* was detected in significant higher number compared to SL1344 6 hours post-infection due to the evasion of pyroptosis and therefore reduced cell death induction.

To analyse the relative importance of TNF-α for extrinsic apoptosis induction in macrophages in more detail, we adjusted our previously established *in vitro* cell death kinetics assay. For this, TNF-α was added to the culture media of BMDMs and the subsequent incorporation of PI over time was measured as indicator of cell death. The addition of TNF-α did not result in BMDM cell death over the analysed time period of 48 hours (**Figure 5.3 C**) indicating that exposure of macrophages to TNF-α alone does not trigger extrinsic apoptosis. To analyse if IFN-γ sensitises BMDMs to TNF-α induced cell death, cells were stimulated with IFN-γ prior to TNF-α exposure. This condition led to the induction of cell death in around 25 % of BMDMs starting at 16 hours post-stimulation. Moreover, the exposure to IFN-γ alone did only induce cell death in 5 % of BMDMs over time. Additionally, we investigated the mechanisms behind IFN-γ enhanced TNF-α mediated apoptosis by analysing if IFN-γ stimulation induces the expression of the death ligand corresponding receptor TNFR1. IFN-γ stimulation of BMDMs led to a noticeable upregulation of this death receptors (**Figure 5.3 D**).

In conclusion, these results imply that the absence of TNF-α does not affect inflammasome-induced PCD *in vitro*. However, TNF-α induces a moderate amount of extrinsic BMDM death that requires previous cell activation by IFN-γ. IFN-γ sensitises BMDMs to extrinsic apoptosis induction mediated by these death ligands, likely by upregulating the expression of TNFR1.

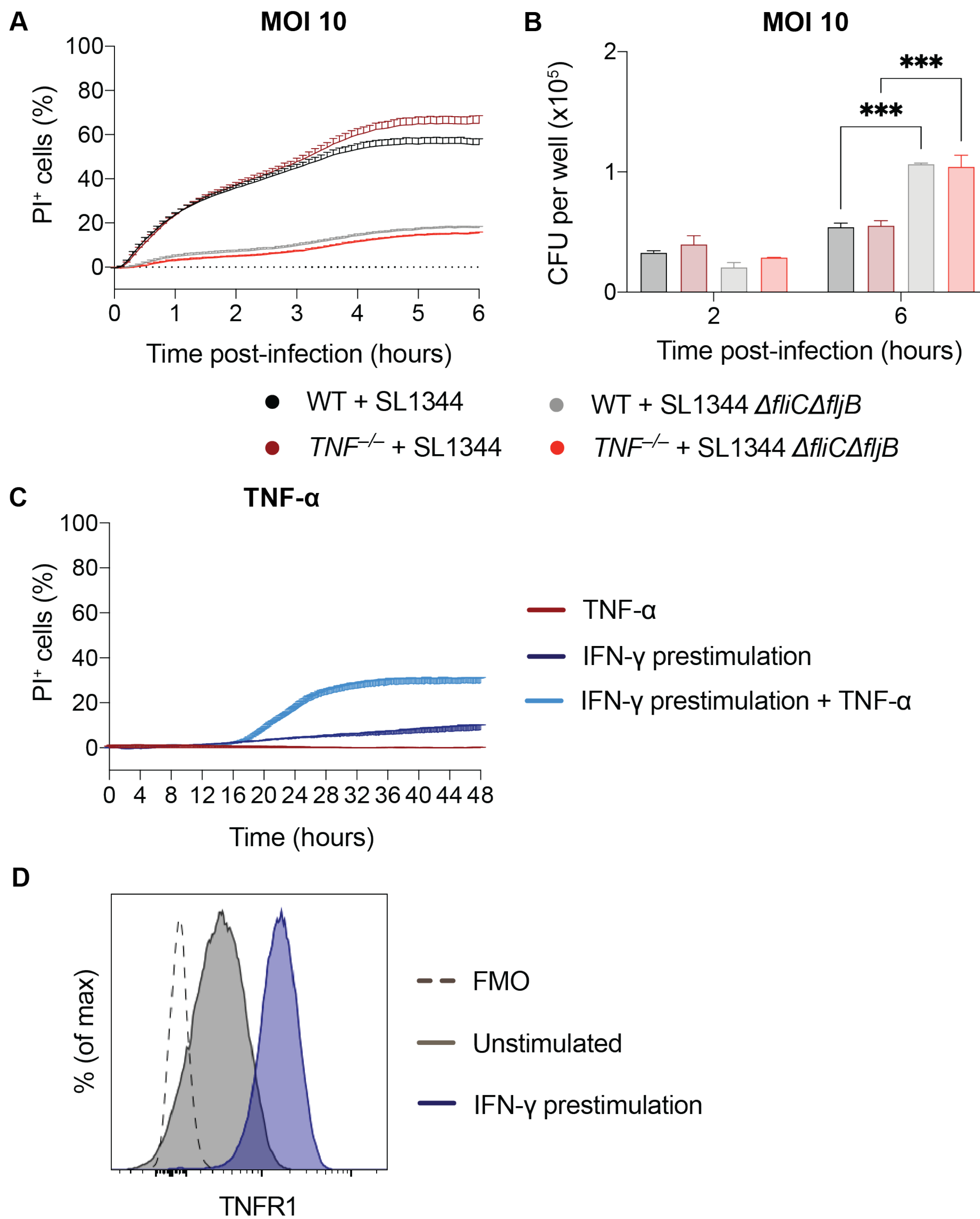

A
MOI 10
PI+ cells (%)
100
80
60
40
20
0
0 1 2 3 4 5 6
Time post-infection (hours)
B
MOI 10
CFU per well (x10^5)
2
1
0
***
***
2
6
Time post-infection (hours)
WT + SL1344
TNF–/– + SL1344
WT + SL1344 ΔfliCΔfljB
TNF–/– + SL1344 ΔfliCΔfljB
C
TNF-α
PI+ cells (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48
Time (hours)
TNF-α
IFN-γ prestimulation
IFN-γ prestimulation + TNF-α
D
% (of max)
TNFR1
FMO
Unstimulated
IFN-γ prestimulation

Figure 5.3: TNF-α is not critical for *Salmonella* Typhimurium control but induces cell death after IFN-γ stimulation of macrophages *in vitro*.
(A and B) Wild-type (WT; depicted in black) and *TNF*$^{-/-}$ (red) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 or SL1344 *ΔfliCΔfljB* (1 hour; MOI 10) followed by gentamicin treatment to remove extracellular bacteria. (A) The incorporation of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. (C) Cell death induced by tumour necrosis factor alpha (TNF-α) was measured via the incorporation of PI of unstimulated and interferon gamma (IFN-γ) prestimulated WT BMDMs over a time period of 48 hours post-exposure. (D) The surface expression of TNF receptor 1 (TNFR1) of unstimulated and IFN-γ prestimulated WT BMDMs was analysed. All experiments were performed once or twice with ≥ 2 technical repeats. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B) and calculated *p*-values are depicted (*** $p < 0.001$). Representative histogram with fluorescence minus one (FMO) is shown (D).

5.2.2 Combined absence of TNF-α, Fas ligand and TRAIL severely impairs control and clearance of *Salmonella* Typhimurium infection *in vivo*

Due to the minor impact of the absence of TNF-α on bacterial titers during *S.* Typhimurium infection *in vivo*, we hypothesised that a functional redundancy of different members of the TNF superfamily for induction of extrinsic apoptosis prevents uncontrolled bacterial growth. Specifically, the expression of FasL and TRAIL could compensate for the loss of TNF-α. An alternative explanation could be that extrinsic apoptosis of target cells is mainly induced through TNF superfamily independent processes, for example the secretion of perforins and granzymes.

To investigate if the combined absence of TNF superfamily members would result in a more pronounced phenotype than TNF-α deficiency alone, we infected *TGLDT*$^{-/-}$ mice, which lack the effector molecules TNF-α, FasL and TRAIL, with 200 CFU of the growth attenuated *S.* Typhimurium strain SL1344 *ΔaroA*. Additionally, *TGLDT*$^{-/-}$ mice were infected with the pyroptosis evading, flagellin deficient strain SL1344 *ΔaroAΔfliCΔfljB* to analyse if this would result in a similar phenotype as previously observed in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice. The infection with either *S.* Typhimurium strain caused significantly elevated bacterial titers in liver and spleen in *TGLDT*$^{-/-}$ mice compared to WT mice 3 weeks post-infection (**Figure 5.4 A**), indicating the importance for TNF-α, FasL and/or TRAIL for control of intracellular infections. Furthermore, *TGLDT*$^{-/-}$ mice infected with the pyroptosis evading flagellin mutant strain SL1344 *ΔaroAΔfliCΔfljB* harboured slightly higher bacterial loads compared to SL1344 *ΔaroA* infected *TGLDT*$^{-/-}$ mice, however this difference did not reach statistical significance. We observed that infected *TGLDT*$^{-/-}$ mice started to lose body weight between week 2 and 3 post-infection (**Figure 5.4 B**). This was more pronounced in the group infected with SL1344 *ΔaroAΔfliCΔfljB*, whose body weights dropped below the initial weight.

Despite that, infected *TGLDT*$^{-/-}$ mice had enlarged livers and spleens compared to WT mice 3 weeks post-infection (**Figure 5.4 C**). The infection with SL1344 *ΔaroAΔfliCΔfljB* did not significantly increase the organ weights of WT and *TGLDT*$^{-/-}$ mice compared to SL1344 *ΔaroA* infection. However, splenomegaly was most pronounced in SL1344 *ΔaroAΔfliCΔfljB* infected *TGLDT*$^{-/-}$ mice.

Interestingly, the combined loss of TNF-α, FasL and TRAIL resulted in higher bacterial titers and more pronounced hepato- and splenomegaly than TNF-α deficiency alone 3 weeks post-infection (**Figure 5.1 A**), implying either a certain level of redundancy between the effector molecules or an important role of FasL and/or TRAIL for the restriction of *S.* Typhimurium. Furthermore, the observed elevated bacterial loads within SL1344 *ΔaroAΔfliCΔfljB* infected *TGLDT*$^{-/-}$ mice almost reached the levels of *S.* Typhimurium BRD509 infected *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice 3 weeks post-infection (**Figure 3.6 A and 3.8**). This suggests that TNF-α, FasL and/or TRAIL are involved in the induction of apoptosis, which becomes most evident in the absence of pyroptosis.

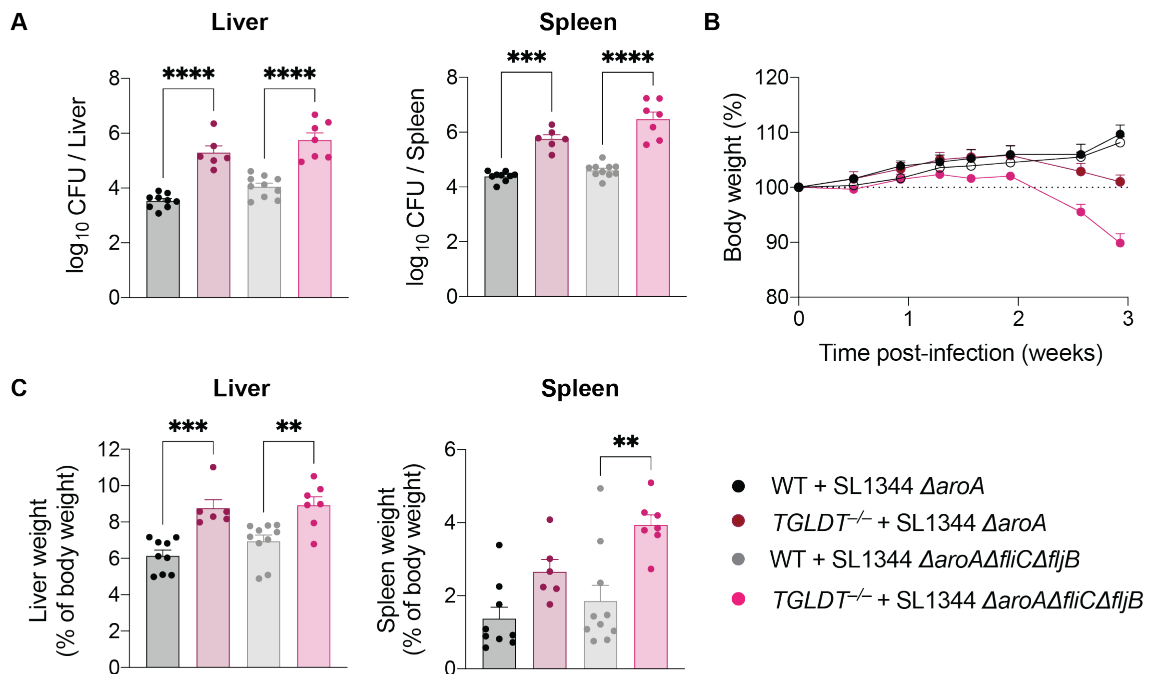


Figure 5.4: Combined absence of TNF-α, Fas ligand and TRAIL results in impaired *Salmonella* Typhimurium control *in vivo*.
Wild-type (WT; depicted in black) and *TGLDT*$^{-/-}$ (magenta) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain SL1344 *ΔaroA* or the growth attenuated, flagellin deficient mutant strain SL1344 *ΔaroAΔfliCΔfljB*. (A) Bacterial titers in the liver and spleen of WT and *TGLDT*$^{-/-}$ mice were determined 3 weeks post-infection. (B) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (C) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. All experiments were performed three times with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by one-way ANOVA (A and C) and calculated *p*-values are depicted (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

We next investigated if the impaired *S.* Typhimurium control observed in *TGLDT*$^{-/-}$ mice would result in the clearance of bacteria with delayed kinetics or if *TGLDT*$^{-/-}$ mice would succumb to infection like *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ mice. Therefore, *TGLDT*$^{-/-}$ mice were infected with 200 CFU of the growth attenuated *S.* Typhimurium strain BRD509 and the course of infection was monitored past 3 weeks post-infection. Infected *TGLDT*$^{-/-}$ mice succumbed to the infection between 3 – 5 weeks post-infection (**Figure 5.5 A**). This was reflected in severely elevated bacterial titers in liver and spleen at the time of sacrifice (**Figure 5.5 B**), loss of body weight starting between week 2 and 3 post-infection (**Figure 5.5 C**) and significant increased livers and spleens at the time of sacrifice compared to WT mice (**Figure 5.5 D**). Overall, the bacterial loads and the relative organ weights of infected *TGLDT*$^{-/-}$ mice further increased past the 3 week post-infection time point (**Figure 5.4 A and C**), indicating that mice were unable to restrict bacterial replication in the combined absence of TNF-α, FasL and TRAIL.

Taken together, these findings indicate that TNF-α, FasL and/or TRAIL are important for *S.* Typhimurium control and clearance *in vivo*. The obtained results suggest that extrinsic apoptosis depends on the presence of TNF superfamily members and implies a certain level of redundancy between them.

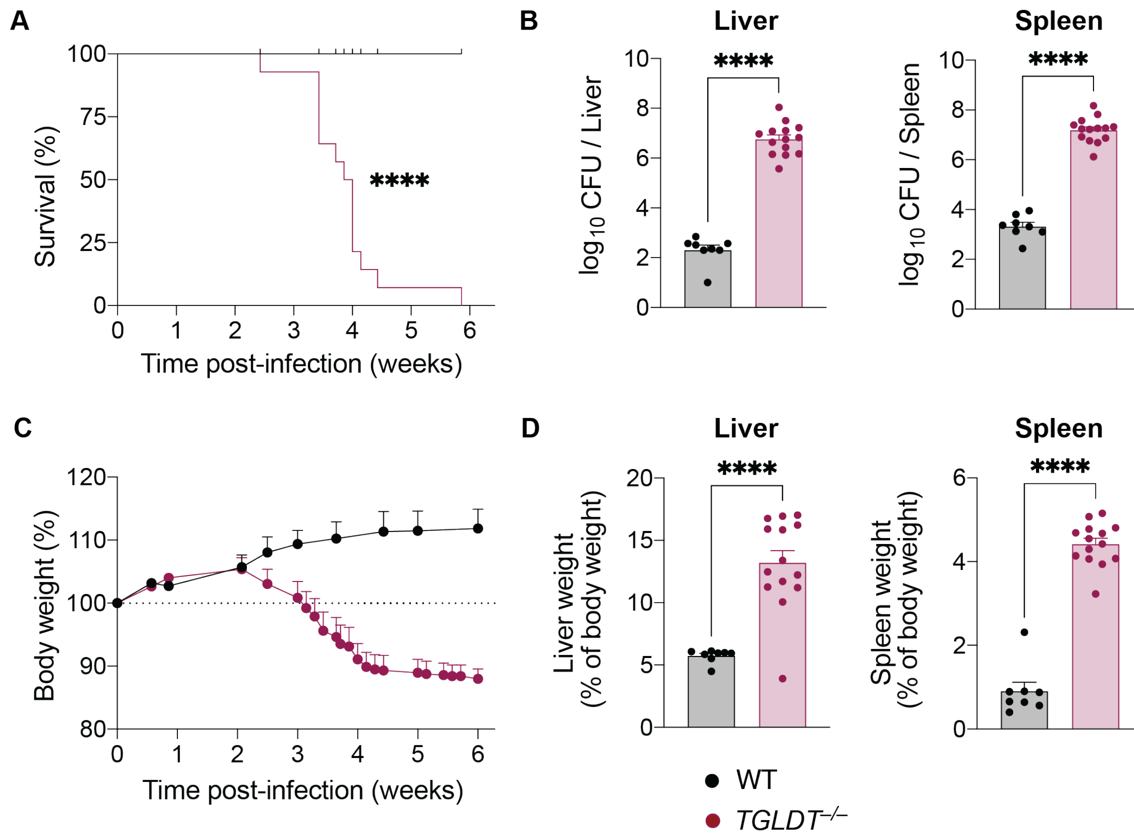


Figure 5.5: The combined loss of TNF-α, Fas ligand and TRAIL leads to impaired *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *TGLDT*$^{-/-}$ (magenta) mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) The survival of infected mice was analysed based on body condition, appearance and behaviour. (B) Bacterial titers in the liver and spleen were determined at the time of sacrifice. (C) The body weight of infected mice was measured and normalised to the body weight on the day of infection. (D) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of sacrifice. All experiments were performed twice with each experimental group including n ≥ 3. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by log rank (Mantel cox) (A) or Student's *t*-test (B and D) and calculated *p*-values are depicted (**** $p < 0.0001$).

To verify the interpretation of these results, we next analysed if the combined deficiency of TNF-α, FasL and TRAIL impacts the induction of inflammasome mediated PCD and *S*. Typhimurium control *in vitro*. Therefore, WT and *TGLDT*$^{-/-}$ BMDMs were infected with MOI 10 of the *S*. Typhimurium strains SL1344 or the non-flagellated strain SL1344 *ΔfliCΔfljB* and cell death kinetics as well as intracellular bacterial burden measured. *TGLDT*$^{-/-}$ BMDMs infected with SL1344 induced pyroptosis with comparable kinetics as WT BMDMs (**Figure 5.6 A**). The overall cell death rate of *TGLDT*$^{-/-}$ BMDMs was slightly lower in comparison to WT BMDMs. However, intracellular bacterial numbers within *TGLDT*$^{-/-}$ and WT BMDMs were similar 2 and 6 hours post-infection (**Figure 5.6 B**). No differences in the cell death kinetics of *TGLDT*$^{-/-}$ and WT BMDMs were detected after infection with the non-flagellated strain SL1344 *ΔfliCΔfljB*, which resulted in the induction of cell death in around 5 % BMDMs (**Figure 5.6 A**). Pyroptosis evasion of the non-flagellated *S*. Typhimurium strain SL1344 *ΔfliCΔfljB* resulted in a significant higher intracellular bacterial burden in *TGLDT*$^{-/-}$ and WT BMDMs 6 hours post-infection compared to infection with SL1344 (**Figure 5.6 B**). Our findings indicated that TNF-α stimulation of BMDM leads to induction of cell death only in combination with IFN-γ. We therefore investigated if BMDMs are more sensitive to FasL induced extrinsic cell death *in vitro*. The stimulation of BMDMs with FasL alone resulted in cell death induction with fast kinetics starting already after 1 hour of exposure (**Figure 5.6 C**). Overall, around 40 % of macrophages succumbed to FasL exposure. The prestimulation of BMDMs with IFN-γ resulted in the faster onset of cell death and increased the overall cell death rate induced by FasL to around 70 %. The stimulation with IFN-γ alone did not results in any BMDM death. Next, we analysed if IFN-γ enhances FasL mediated apoptosis by inducing the expression of the death receptors Fas. We detected pronounced levels of Fas expression on the surface of unstimulated BMDMs, which was considerably upregulated by IFN-γ stimulation (**Figure 5.6 D**). We noticed that the observed BMDM cell death induced by FasL occurred with very fast kinetics similar to pyroptosis induction in *S.* Typhimurium infected BMDMs. To verify that FasL dependent cell death is mediated by caspase-8, we

exposed *Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ BMDMs to this death receptor ligand. The cell death induced by FasL in WT BMDMs was completely absent in caspase-8 deficient BMDMs (**Figure 5.6 E**). Recent reports indicate that caspase-8 cleaves the pyroptotic cell death effector GSDMD (Demarco et al., 2020, Gram et al., 2019, Orning et al., 2018, Sarhan et al., 2018), which could result in faster cell death execution compared to initiating the apoptotic caspase cascade. To investigate if caspase-8 induced BMDM cell death via GSDMD cleavage downstream of FasL stimulation, *GsdmD*$^{-/-}$ macrophages were exposed to FasL. Interestingly, *GsdmD*$^{-/-}$ BMDMs died with delayed kinetics and showed a slightly reduced overall cell death rate compared to WT BMDMs. Moreover, the cell death kinetics of *GsdmD*$^{-/-}$ BMDMs were similar to the induction of apoptosis in pyroptosis deficient BMDMs infected with *S.* Typhimurium (**Figure 3.13 A**).

In conclusion, these findings indicate that extrinsic apoptosis of BMDMs can be induced by FasL. IFN-γ is not necessary but promotes FasL mediated cell death of BMDMs, likely by upregulation of the expression of Fas. Overall, FasL seems to be an effective macrophage cell death inducing molecule by initiating downstream caspase-8 and GSDMD activation.

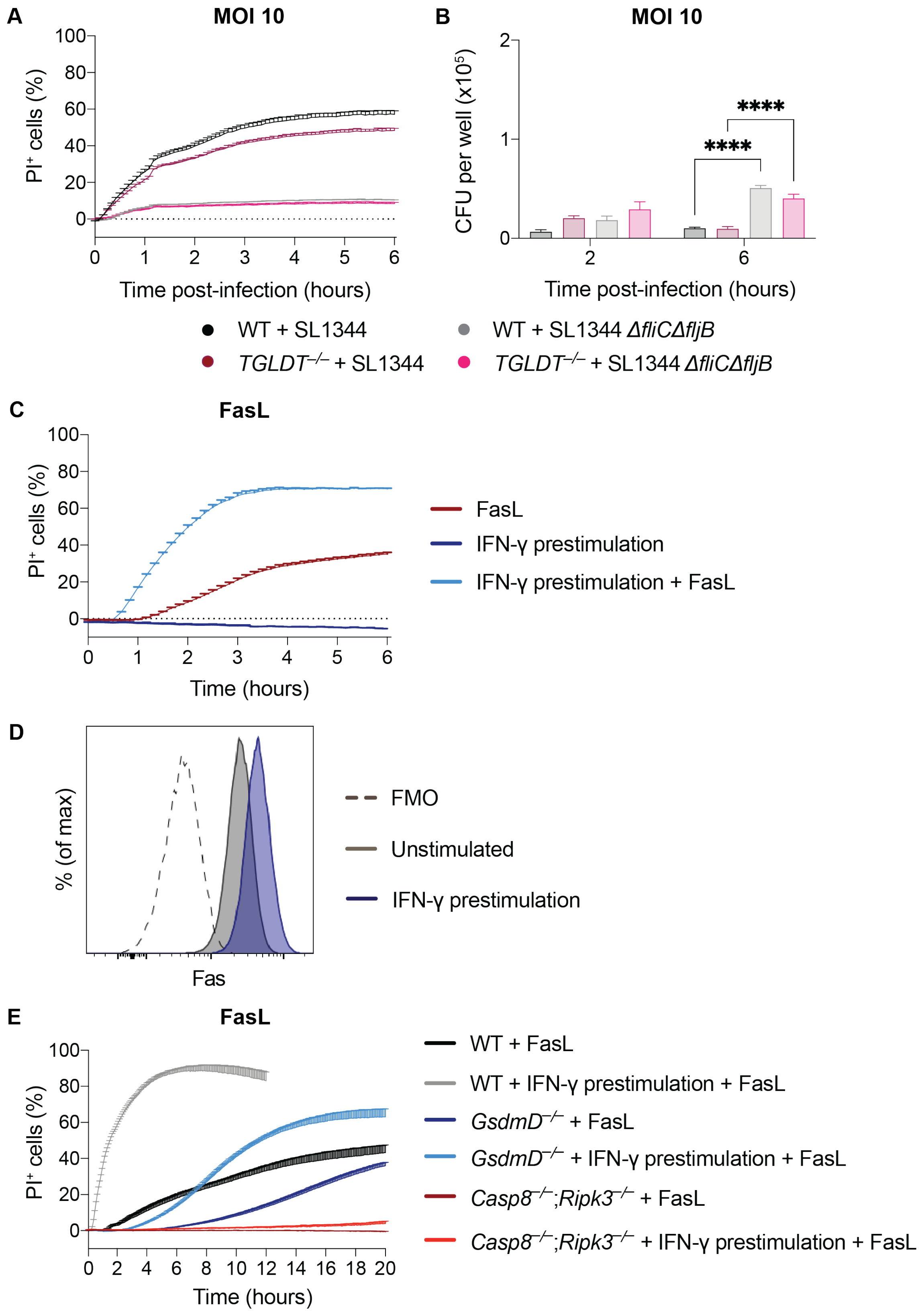

A
MOI 10
PI⁺ cells (%)
Time post-infection (hours)
B
MOI 10
CFU per well (x10⁵)
****
****
Time post-infection (hours)
WT + SL1344
WT + SL1344 ΔfliCΔfljB
TGLDT−/− + SL1344
TGLDT−/− + SL1344 ΔfliCΔfljB
C
FasL
PI⁺ cells (%)
Time (hours)
FasL
IFN-γ prestimulation
IFN-γ prestimulation + FasL
D
% (of max)
Fas
FMO
Unstimulated
IFN-γ prestimulation
E
FasL
PI⁺ cells (%)
Time (hours)
WT + FasL
WT + IFN-γ prestimulation + FasL
GsdmD−/− + FasL
GsdmD−/− + IFN-γ prestimulation + FasL
Casp8−/−;Ripk3−/− + FasL
Casp8−/−;Ripk3−/− + IFN-γ prestimulation + FasL

Figure 5.6: TNF-α, Fas ligand and TRAIL are not crucial for *Salmonella* Typhimurium induced cell death and IFN-γ sensitises macrophages to Fas ligand induced programmed cell death *in vitro*.
(A and B) Wild-type (WT; depicted in black) and *TGLDT*$^{-/-}$ (magenta) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 or SL1344 *ΔfliCΔfljB* (1 hour; MOI 10) followed by gentamicin treatment to remove extracellular bacteria. (A) The incorporation of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 hours post-infection. (B) Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. (C) Cell death induced by Fas ligand (FasL) was measured via the incorporation of PI of unstimulated and interferon gamma (IFN-γ) prestimulated WT BMDMs over a time period of 48 hours post-exposure. (D) The surface expression of Fas of unstimulated and IFN-γ prestimulated WT BMDMs was analysed. (E) Unstimulated and IFN-γ prestimulated WT (black), *GsdmD*$^{-/-}$ (blue) and *Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ (red) immortalised BMDMs (iBMDMs) were exposed to FasL. Cell death induced by FasL was measured via the uptake of PI of iBMDMs over a time period of 12 hours post-exposure. All experiments were performed once or twice with ≥ 2 technical repeats. Data were pooled and are expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (B) and calculated *p*-values are depicted (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). Representative histogram with fluorescence minus one (FMO) is shown (D).

5.2.3 CD4^{+} T cells are important for induction of extrinsic apoptosis during *Salmonella* Typhimurium infection *in vivo*

Our results indicate that TNF-α, FasL and/or TRAIL are critical for the induction of apoptosis and for control and clearance of *S.* Typhimurium infection *in vivo*. One lymphocyte subset that is essential for clearing intracellular infections and can secrete the mentioned effector molecules are CD4^{+} T cells. Diminished extrinsic apoptotic cell death induction in infected cells due to the lack of functional CD4^{+} T cells could explain the findings why CD4^{+} T cell deficient mice are unable to clear *S.* Typhimurium infections. We therefore investigated the involvement of CD4^{+} T cells in the initiation of apoptosis in the immune response against *S.* Typhimurium infections.

We therefore infected WT and caspases-1, -11 and -12 deficient mice, which rely on apoptosis to control *S.* Typhimurium, with 200 CFU of the growth attenuated strain BRD509 and analysed bacterial titers at week 7 and 12 post-infection within the T cell-dependent phase of the immune response. As previously shown (**Figure 3.2 A**), WT and pyroptosis deficient mice clear the infection with the attenuated *S.* Typhimurium strain BRD509 from liver and spleen within 12 weeks (**Figure 5.7 A**) without signs of extensive hepato- and splenomegaly (**Figure 5.7 B**) or weight loss at the analysed time points (**Figure 5.7 C**). CD4^{+} T cells were depleted by intraperitoneal injection of the monoclonal antibody GK1.5 twice weekly and the efficiency of depletion (>95 %) was confirmed via FACS (data not shown). As published previously (Kupz et al., 2014), the depletion of the CD4^{+} T cell subset resulted in the development of a chronic infection in WT mice indicated by prolonged detection of bacterial titers in liver and spleen until 12 weeks post-infection compared to the PBS treated control group (**Figure 5.7 A**). Relative liver and spleen weights of CD4^{+} T cell depleted WT mice were not significantly increased (**Figure 5.7 B**) and no weight loss was detected (**Figure 5.7 C**). This indicates that CD4^{+} T cell-independent host immune mechanisms, such as pyroptosis, prevent uncontrolled growth of *S.* Typhimurium bacteria. Interestingly, the bacterial titers of CD4^{+} T cell depleted *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$ mice

were significantly higher 7 and 12 weeks post-infection compared to those of WT mice lacking CD4$^+$ T cell (**Figure 5.7 A**). Additionally, we observed a significant increase in liver and spleen bacterial numbers in CD4$^+$ T cell depleted pyroptosis deficient mice between week 7 and 12 post-infection, in contrast to the control groups, which have either decreasing or stable bacterial titers over time (**Figure 5.7 A**). This was also reflected by significantly enlarged livers and spleens at week 12 post-infection (**Figure 5.7 B**). Moderate physical signs of distress and reduced responsiveness indicated that CD4$^+$ T cell depleted pyroptosis deficient mice developed severe disease. However, CD4$^+$ T cell depleted *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$ mice did not lose body weight (**Figure 5.7 C**), which might have been partially compensated for by the extensive hepato- and splenomegaly.

Taken together these results indicate that CD4$^+$ T cells are critical for extrinsic apoptosis during *S.* Typhimurium infection. Strikingly, apoptosis, which can act as functional backup for pyroptosis and effective immune defence mechanism against *S.* Typhimurium, seems to not be sufficient in the absence of CD4$^+$ T cells.

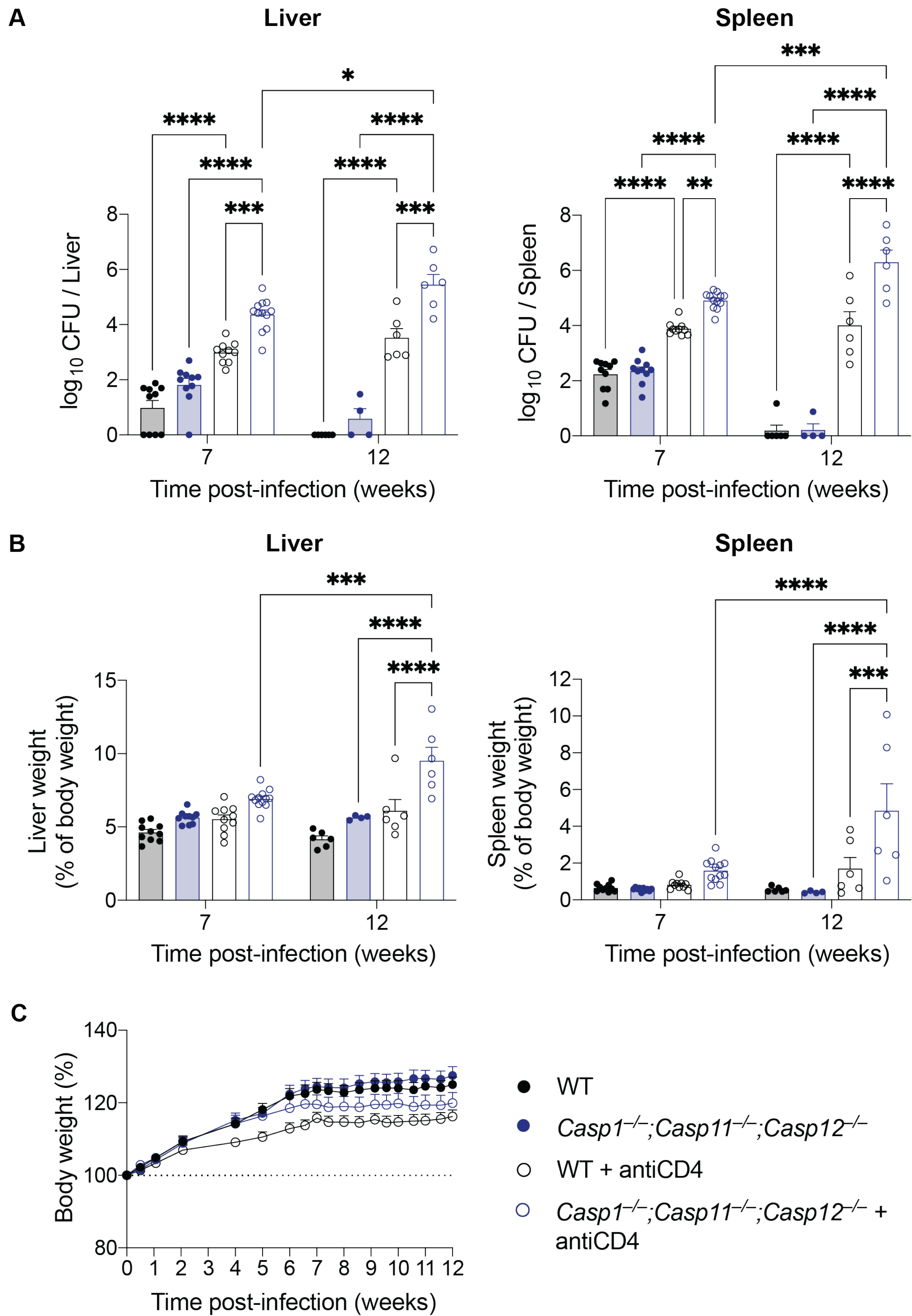

A
Liver
Spleen
log10 CFU / Liver
log10 CFU / Spleen
Time post-infection (weeks)
B
Liver weight (% of body weight)
Spleen weight (% of body weight)
C
Body weight (%)
WT
Casp1–/–;Casp11–/–;Casp12–/–
WT + antiCD4
Casp1–/–;Casp11–/–;Casp12–/– + antiCD4

Figure 5.7: CD4$^+$ T cell depletion leads to impaired *Salmonella* Typhimurium clearance *in vivo*.
Wild-type (WT; depicted in black) and *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$* (blue) mice were injected intraperitoneally with GK1.5.7 (antiCD4; open circles) or phosphate buffered saline (PBS; closed circles) on day -2; -1 and then twice weekly and infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. (A) Bacterial titers in the liver and spleen of WT and *Casp1$^{-/-}$;Casp11$^{-/-}$;Casp12$^{-/-}$* mice were determined 7 and 12 weeks post-infection. (B) Liver and spleen weights of infected mice were measured and normalised to the body weight on the day of analysis. (C) The body weight of infected mice was measured and normalised to the body weight on the day of infection. All experiments were performed twice with each experimental group including $n \geq 2$. Data were pooled and expressed as mean + SEM. Statistically significant differences were determined by two-way ANOVA (A and B) and calculated *p*-values are depicted (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

5.2.4 $CD4^+$ T cells effectively induce cell death of macrophages via Fas ligand in a dose-dependent and antigen-specific manner *in vitro*

As macrophages are known to be one of the main targets of *S.* Typhimurium to persist in, we investigated the efficiency and mechanisms of $CD4^+$ T cell induced macrophage killing in a more direct approach. $CD4^+$ T cells recognise antigen presented via MHC-II molecules on the surface of APCs using their TCRs. Therefore, WT BMDMs were pulsed with the HSV-1 glycoprotein D and incubated with TCR transgenic $CD4^+$ T cells (gDT-II cells) specific for the gD-derived epitope $gD_{315-327}$. BMDM cell death induced by $CD4^+$ T cells was measured via the incorporation of PI *in vitro*.

Activated $CD4^+$ T cells killed BMDMs in a dose-dependent and antigen-specific manner (**Figure 5.8 A**). BMDMs that were not pulsed with the gD peptide before exposure to activated gDT-II cells in a ratio of 1:2.5 – 1:7.5 did not incorporate PI in the analysed time period. In contrast to that, BMDMs loaded with the gD peptide prior to co-culture with $CD4^+$ T cells did incorporate PI. We observed the induction of BMDM cell death after 3 – 4 hours of co-culture with $CD4^+$ T cells. A higher PI fluorescence signal was detected with increasing numbers of added $CD4^+$ T cells, implying that $CD4^+$ T cells induced BMDM cell death in a dose dependent manner. We next analysed the relative importance of the $CD4^+$ T cell effectors TNF-α, FasL and TRAIL for $CD4^+$ T cell induced BMDM apoptosis by addition of blocking antibodies (**Figure 5.8 B**). TNF-α inhibition did not reduce the observed $CD4^+$ T cell killing of BMDMs, while inhibition of TRAIL led to a slight reduction in the overall macrophage death rate. Interestingly, the blocking of FasL did completely inhibit $CD4^+$ T cell induced BMDM death to a similar extend than the combined blocking of all three effectors.

Overall, these results indicate that antigen specific $CD4^+$ T cells effectively induce macrophage cell death. Furthermore, our findings imply that FasL is the main cytotoxic effector of $CD4^+$ T cell mediated macrophage killing.

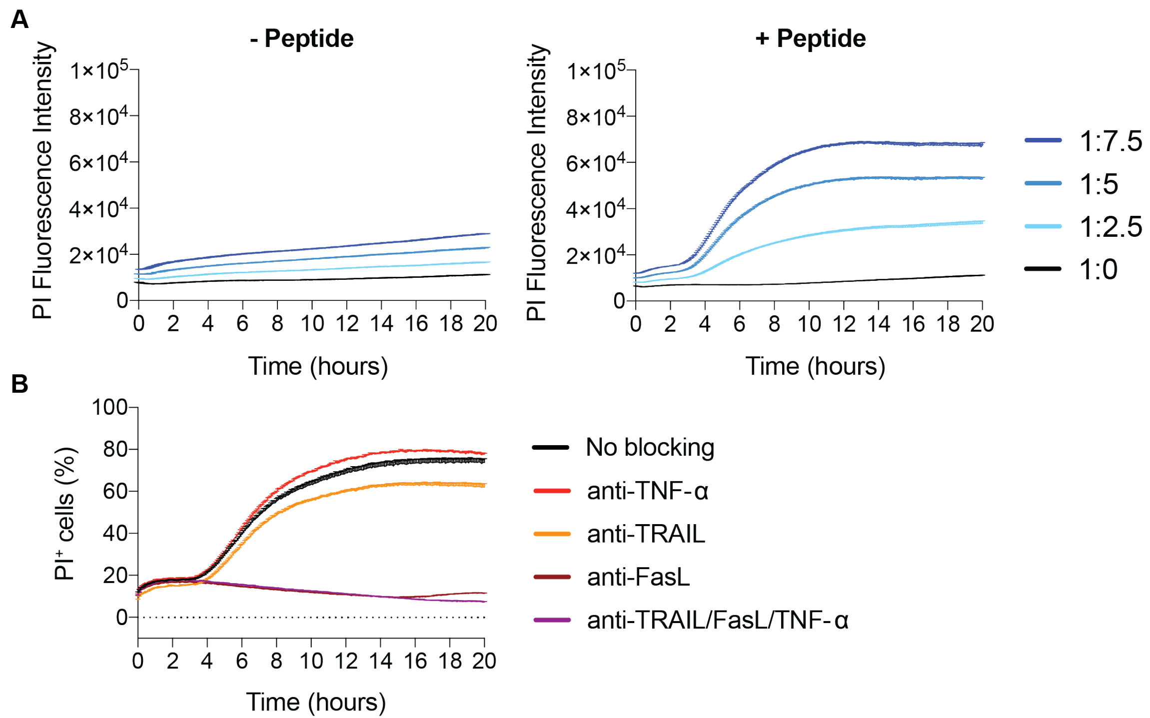


Figure 5.8: CD4⁺ T cell mediated macrophages killing *in vitro* is mediated by Fas ligand.

(A) Non pulsed and (A and B) glycoprotein D pulsed wild-type (WT) bone marrow-derived macrophages (BMDMs) were co-cultured together with T cell receptor transgenic $CD4^+$ T cells. (A) $CD4^+$ T cell induced BMDM cell death (1:0 – 1:7.5 target cell to effector cell ratio) was measured via the uptake of propidium iodide (PI; a marker of cell death) over a time period of 20 hours. (B) Blocking antibodies for tumour necrosis factor (TNF-α), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) were added alone or in combination and $CD4^+$ T cell induced WT BMDM cell death (1:5 target cell to effector cell ratio) was measured via the uptake of PI over a time period of 20 hours. All experiments were performed once with ≥ 2 technical repeats. Data were pooled and are expressed as mean + SEM.

5.2.5 *Salmonella* Typhimurium infection interferes with CD4$^+$ T cell mediated killing and MHC-II expression on macrophages

S. Typhimurium interferes with multiple host cell processes, including antigen presentation. Previous studies implied that the SPI-2 effector molecule SteD causes host E3 ubiquitin ligase MARCH8 mediated ubiquitination and depletion of mature MHC-II on the surface of infected APCs (Bayer-Santos et al., 2016).

Therefore, we determined if *S.* Typhimurium interferes with the CD4$^+$ T cells mediated killing of infected macrophages. We infected BMDMs with the *S.* Typhimurium strain SL1344 *ΔinvA* to prevent the rapid induction of inflammasome driven PCD. Surprisingly, BMDMs infected with *S.* Typhimurium prior to the co-culture with CD4$^+$ T cells were partially resistant to extrinsic apoptosis induction (**Figure 5.9 A**). One possible explanation for this could be the finding that *S.* Typhimurium infection interferes with the surface levels of MHC-II molecules on infected BMDMs (**Figure 5.9 B**). This observation was enhanced when MHC-II expression of BMDMs was induced by IFN-γ stimulation prior to infection.

To analyse the effect of *S.* Typhimurium infection on the expression of MHC-II on macrophages *in vivo*, we infected WT mice with 200 CFU of BRD509 and compared the splenic monocytes and macrophages 3 weeks post-infection to that of uninfected WT mice. Two populations with different expression levels of MHC-II were detected in uninfected WT mice, indicating that distinct subsets of splenic monocytes/macrophages express varying amounts of MHC-II, which could be reflective of their activation status (**Figure 5.9 C**). The expression of MHC-II on monocytes/macrophages was high in WT mice following infection with *S.* Typhimurium. However, this seemed to be dependent on IFN-γ, as monocytes/macrophages of *IFN-*$\gamma^{-/-}$ mice showed a pronounced decrease in MHC-II expression after infection.

Taken together, our results imply that *S.* Typhimurium infection seems to interfere with the MHC-II surface expression of macrophages and thereby inhibit recognition by $CD4^+$ T cells and downstream apoptosis induction. Furthermore, the secretion of IFN-γ during *S.* Typhimurium infection regulates the expression of MHC-II on infected macrophages *in vivo*.

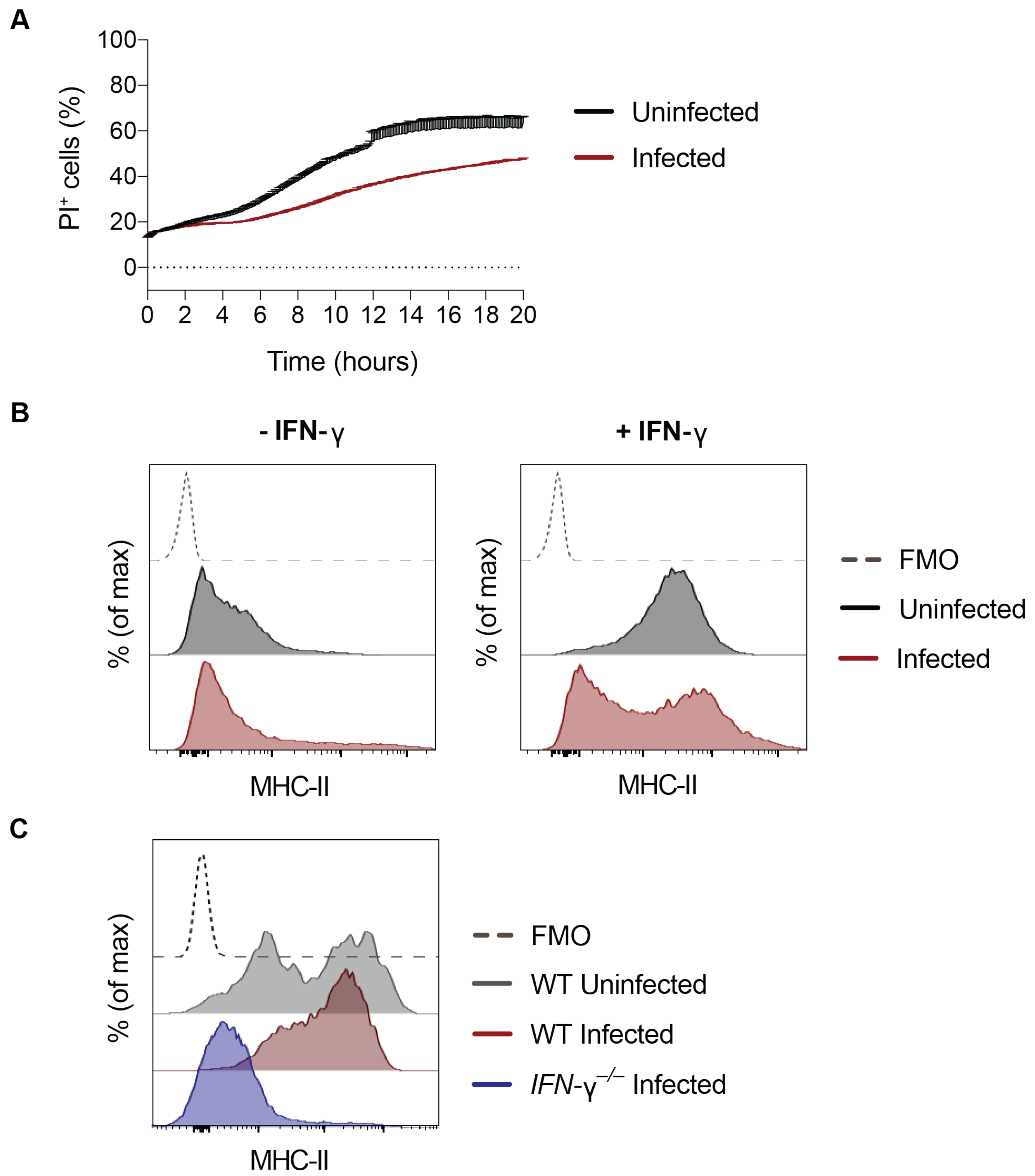

A
PI⁺ cells (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
Time (hours)
Uninfected
Infected
B
- IFN-γ
+ IFN-γ
% (of max)
MHC-II
FMO
Uninfected
Infected
C
% (of max)
MHC-II
FMO
WT Uninfected
WT Infected
IFN-γ−/− Infected

Figure 5.9: *Salmonella* Typhimurium infection interferes with CD4$^+$ T cell mediated macrophage killing and MHC-II expression.
(A) Wild-type (WT) bone marrow-derived macrophages (BMDMs) were uninfected or infected with the *Salmonella* Typhimurium strain SL1344 *ΔinvA* (MOI 50; overnight) prior to glycoprotein D pulsing and co-culture with T cell receptor transgenic CD4$^+$ T cells (1:5 target cell to effector cell ratio). CD4$^+$ T cell induced BMDM cell death was measured via the uptake of propidium iodide (PI; a marker of cell death) over a time period of 20 hours. (B) The surface expression of major histocompatibility complex (MHC)-II of unstimulated and interferon gamma (IFN-γ) prestimulated WT BMDMs was analysed. BMDMs were uninfected or infected with SL1344 *ΔinvA* (MOI 10; overnight). (C) WT and *IFN-γ*$^{-/-}$ mice were infected intravenously with 200 colony forming units (CFU) of the growth attenuated *Salmonella* Typhimurium strain BRD509. The surface expression of MHC-II on viable splenic monocytes/macrophages (CD3$^-$CD19$^-$NK1.1$^-$Ly6G$^-$CD64$^+$) was analysed 3 weeks post-infection. All experiments were performed once with ≥ 2 technical repeats. Data were pooled and are expressed as mean + SEM. Representative histograms with fluorescence minus one (FMO) are shown (B and C).

5.3 Discussion

In this chapter we described novel roles for $CD4^+$ T cells and IFN-γ during *S.* Typhimurium infections, besides their established helper function and antimicrobial effects respectively. We examined the intriguing hypothesis that IFN-γ and $CD4^+$ T cells promote and execute apoptotic cell death of infected macrophages. Our results indicate that IFN-γ induces the expression of the death receptors TNFR1 and Fas as well as MHC-II on macrophages *in vitro*, thereby sensitising them to extrinsic apoptosis induction. Furthermore, we showed that antigen-specific $CD4^+$ T cells effectively kill macrophages via FasL and downstream caspase-8 activation. These new insights could explain previous findings that $CD4^+$ T cells and MHC-II expression are required for the efficient clearance of *S.* Typhimurium infections *in vivo*.

Following host cell invasion, *S.* Typhimurium remodels its surface extensively to avoid detection by cellular sensors, limit antigen availability and presentation as well as inflammasome assembly and cell death induction. This includes the downregulation of flagella, which are not required within the host cell (Cummings et al., 2006, Miao et al., 2010a, Lai et al., 2013, Sporing et al., 2018). Intracellular *S.* Typhimurium also downregulate the SPI-1 T3SS, which is important for invasion, and instead upregulate the SPI-2 T3SS that facilitates survival within the host cell (Cirillo et al., 1998, Galan, 1999, Galan and Collmer, 1999, Galan and Curtiss, 1989, Galan and Zhou, 2000, Hensel et al., 1998, Mills et al., 1995, Ochman et al., 1996, Shea et al., 1996). In mice, flagella, the SPI-1 T3SS needle protein PrgI and inner rod protein PrgJ are sensed by cytosolic sensors of the NAIP family (Kofoed and Vance, 2011, Rauch et al., 2016, Rayamajhi et al., 2013, Yang et al., 2013, Zhao et al., 2016, Zhao et al., 2011). In contrast, the SPI-2 T3SS inner rod protein SsaI is not sensed by NAIPs, which allows *S.* Typhimurium to evade detection and induction of pyroptosis (Miao et al., 2010b, Reyes Ruiz et al., 2017). The upregulation of SPI-2 within human macrophages was implied to supress the translocation of SPI-1 effector molecules and thereby acts as immune evasion strategy of *S.* Typhimurium to

subvert NLRP3 and NLRC4 inflammasome responses (Bierschenk et al., 2019). A recent study investigated inflammasome signalling during chronic *S.* Typhimurium infection (Cai et al., 2022). The findings implied that the initial pathogen control is facilitated by upregulated inflammasome activation and induction of pyroptosis. However, in the course of infection as *S.* Typhimurium persists, modulation of SPI-1 and flagella resulted in the reduction of inflammasome activation. Therefore, infected cells may switch towards the induction of other types of PCD. Furthermore, *S.* Typhimurium induces protective resistance mechanisms regulated by the two-component regulatory PhoP/PhoQ system to counteract intracellular nutrient limitation and digestion (Bourret et al., 2017, Vazquez-Torres and Fang, 2001). All strategies combined allow *S.* Typhimurium to evade host cell pyroptosis, avoid the inactivation by cellular ROS and NO and result in the establishment of the intracellular replicative niche within modified phagosomes.

The induction of apoptosis most likely enables the host to counteract the above-mentioned multiple strategies of *S.* Typhimurium to evade pyroptosis as well as control and clear the infection. We hypothesise that this failsafe system is orchestrated by $CD4^+$ T cells, as $CD4^+$ T cell deficient mice were not able to resolve infection with growth attenuated *S.* Typhimurium (Hess et al., 1996, Kupz et al., 2014). Furthermore, in the chronic *S.* Typhimurium infection model it was shown that bacteria persist in granuloma macrophages that could not interact with Th1 cells (Goldberg et al., 2018). These findings are in line with our results indicating that cytotoxic $CD4^+$ T cells are required for the induction of extrinsic apoptosis in infected host cells.

However, *S.* Typhimurium can not only inhibit pyroptotic cell death but can also avoid the induction of apoptosis. It was previously shown that *S.* Typhimurium interferes with the MHC-II expression on APCs (Godlee et al., 2022, Gogoi et al., 2018, Lapaque et al., 2009, Mitchell et al., 2004). Specifically, the SPI-2 T3SS dependent *S.* Typhimurium effector SteD causes host E3 ubiquitin ligase MARCH8 mediated ubiquitination and depletion of mature MHC-II on the surface

of infected APCs (Bayer-Santos et al., 2016). This resulted in reduced T cell activation during *S.* Typhimurium infection *in vitro* and *in vivo*. Our results confirm that *S.* Typhimurium interferes with the surface MHC-II expression of infected macrophages and additionally indicate that *S.* Typhimurium thereby inhibits $CD4^+$ T cell induced killing of target cells.

The activation of infected cells by IFN-γ seems to be an effective host immune mechanism to counteract apoptosis evasion by *S.* Typhimurium and sensitise macrophages to $CD4^+$ T cell induced cell death. $CD4^+$ T cells themselves are an important source of IFN-γ during *S.* Typhimurium infection. It was previously reported that IFN-γ increases the expression of Fas on murine and human macrophages (Ashany et al., 1995). Furthermore, IFN-γ was shown to prime macrophages to TLR and TNF-α induced cell death (Karki et al., 2021, Simpson et al., 2022).

Our results indicate that cytotoxic $CD4^+$ T cells predominately kill macrophages via Fas/FasL, which verifies previous results (Ashany et al., 1995). TNF-α and TRAIL seem to play no critical role in extrinsic apoptosis induction. One possible explanation for that are the various roles of TNF-α, including the involvement in cell survival and activation. Ligation of TNF-α to TNFR1 promotes pro-inflammatory and antimicrobial gene transcription through the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways (Arthur and Ley, 2013, Liu et al., 2017, Sun, 2017). The outcomes of this signalling cascade are the expression of pro-survival proteins, including the caspase-8 inhibitor cellular FLICE inhibitory protein (cFLIP) (Webster and Vucic, 2020). Previous reports imply that TNF-α stimulation of macrophages is important to promote their pro-inflammatory phenotype and limit the effects of the *S.* Typhimurium effector SteE, which facilitates an anti-inflammatory phenotype of macrophages (Pham et al., 2020). Confirming our findings, studies showed that TNF-α triggers PCD only in specific situations, for example when secreted together with IFN-γ via the JAK/STAT1/IRF1 axis (Karki et al., 2021).

Previous studies investigated the impact of TRAIL receptor deficiency during intracellular infection. The lack of TRAIL-R did not increase the bacterial numbers in liver and spleen of *Listeria monocytogenes* infected mice or reduce the survival after *S.* Typhimurium 14028 infection compared to WT mice (Diehl et al., 2004). Furthermore, no significant differences in the bacterial burden in liver and spleen of *S.* Typhimurium BRD509 infected *TRAIL-R*$^{-/-}$ mice was observed 3 weeks post-infection compared to WT mice (Newson et al., 2019). These results indicate that TRAIL signalling plays no major role in the regulation of PCD during infection with *S.* Typhimurium, which is in line with our *in vitro* findings implying that TRAIL is not essential for cytotoxic CD4^{+} T cell induced macrophage killing.

Our findings are in line with previous publications that investigated macrophage death following infections with the intracellular bacterium *Mycobacterium tuberculosis* (Bocchino et al., 2005). These studies revealed that human monocyte-derived macrophages are sensitive to FasL and TNF-α induced apoptosis when stimulated with cycloheximide (Oddo et al., 1998). Similar to *S.* Typhimurium, *Mycobacterium tuberculosis* interferes with cytotoxic CD4^{+} T cell induced target cell death by interfering with antigen presentation (Bocchino et al., 2005). The infection of macrophages with *Mycobacterium tuberculosis* resulted in decreased levels of Fas expression and reduced susceptibility to FasL induced cell death (Oddo et al., 1998). Further studies revealed lower expression of the costimulatory molecules and MHC-II on *Mycobacterium tuberculosis* infected macrophages (Gercken et al., 1994, Saha et al., 1994).

There is only limited data about the role of cytotoxic CD4^{+} T cell and FasL induced apoptosis *in vivo*. It was reported that mice deficient for FasL harbour a significantly decreased CD4^{+} T cell mediated cytotoxicity (Lowin et al., 1994). Furthermore, mice lacking FasL fail to eliminate infections with the intracellular parasite *Leishmania* major, which can be rescued by FasL injections (Conceicao-Silva et al., 1998). A more recent report indicates that FasL deficient mice had significantly increased bacterial titers following *Mycobacterium tuberculosis*

infection (Stutz et al., 2021). However, mice lacking TNF-α displayed an even more pronounced phenotype, which implies that TNF-α plays a predominant role in control of *Mycobacterium tuberculosis* infections *in vivo* (Stutz et al., 2021). The importance of TNF-α for immunity against *Mycobacterium tuberculosis* was also observed in humans. The use of anti TNF agents, used in individuals to treat various inflammatory or autoimmune diseases, has been linked to reactivation tuberculosis (Ehlers, 2003, Harris and Keane, 2010).

It is unclear why the immune responses against *S.* Typhimurium and *Mycobacterium tuberculosis* differ in their reliance on TNF-α. One possible explanation could be that both pathogens have developed different strategies to interfere with the immune system and persist within the host (Tischler and McKinney, 2010), suggesting that specific cytokines and immune responses are more important for the control and clearance of one pathogen than another. Additionally, recent findings indicate that FasL mediated cytotoxicity of $CD4^+$ T cells is a critical immune mechanism during chronic retrovirus infection (Malyshkina et al., 2017). Besides that, our group revealed that cytotoxic $CD4^+$ T cells effectively kill melanoma cells through Fas/FasL signalling (unpublished data). All findings combined argue for an important role for cytotoxic $CD4^+$ T cells in the regulation of PCD through mechanisms including the expression of FasL during infectious diseases and cancer.

It would be interesting to analyse if *S.* Typhimurium infection interferes with the expression of costimulatory molecules, important for interaction with $CD4^+$ T cell, and members of the TNF receptor superfamily, especially Fas, on infected BMDMs in addition to the observed inhibitory effects on MHC-II. The published data on that matter is contradictory, most likely because different cell lines and *S.* Typhimurium strains were analysed. While one study indicated upregulation of CD80 and CD86 on J774 macrophages following infection with *S.* Typhimurium (Kalupahana et al., 2005), other studies reported unchanged levels of CD80 and CD86 on THP-1 cells (Mitchell et al., 2004) or suggested that *S.* Typhimurium reduces the expression of CD86 on BMDCs via its effector SteD (Bayer-Santos

et al., 2016). Rosenberger and colleagues showed that *S.* Typhimurium or LPS treatment of RAW 264.7 macrophages results in elevated mRNA levels of signalling molecules involved in cell death, including TNFR1, Fas and TRAIL (Rosenberger et al., 2000).

Infection of Fas or FasL deficient mice with *S.* Typhimurium would provide further insights about the importance of Fas/FasL during intracellular infection *in vivo*. Published findings indicate that Fas deficient mice were more susceptible to *Listeria monocytogenes* infection resulting in a significant higher bacterial burden in the liver of infected *Fas*$^{-/-}$ mice compared to WT mice (Uchiyama et al., 2013).

Our *in vivo* data suggests that different subsets of monocyte and macrophage express distinct levels of Fas and MHC-II depending on their activation and infection status. Additional experiments investigating the individual subsets via markers such as CD11b, CD11c, Ly6C and MerTK and their expression profile in detail could provide further insights if *S.* Typhimurium predominately persists in cell subsets with reduced expression of MHC-II and cell death receptor. Additionally, a detailed analysis of the dynamics between the cell death promoting effects of IFN-γ and cell death inhibitory effects of *S.* Typhimurium is necessary. Furthermore, infection of mixed bone-marrow chimeric mice in which apoptosis can only be induced in MHC-II deficient cells would be a complementary approach to the presented depletion experiments and could provide further insights into the requirement of CD4^{+} T cells for extrinsic apoptosis induction during intracellular infections. Even though Fas ligand seems to be the main initiator of CD4^{+} T cell mediated macrophage extrinsic apoptosis, it is necessary to investigate the importance of the perforin/granzyme pathway, which is commonly described as the most potent effector function of cytotoxic CD8^{+} T cells. Previous studies suggested that perforin-2 breaches the envelope of phagocytosed *S.* Typhimurium, which facilitates the delivery of antimicrobial effectors into the bacteria (Bai et al., 2018). Granzyme B was reported to attenuate bacterial virulence by degrading multiple effector proteins from *S.* Typhimurium, *Listeria monocytogenes* and *Mycobacteria tuberculosis* (Leon

et al., 2020). Perforin, granzyme B and perforin/granzyme B deficient mice infected with *S.* Typhimurium BRD509 showed a slightly impaired bacterial control 3 weeks post-infection but cleared the infection like WT mice within 60 days (Lee et al., 2012). Taken together, these finding argue against a major role for perforin and granzymes in the clearance of primary *S.* Typhimurium infection and strengthen our findings about the importance of Fas/FasL and $CD4^+$ T cell induced apoptosis during *S.* Typhimurium infection.

Chapter 6:

Overall Discussion

6.1 The importance of programmed cell death in health and disease

PCD describes the genetically regulated process of cellular destruction that is induced, controlled and executed by specialised molecular machineries (Galluzzi et al., 2018). The mechanisms of PCD are fundamental for life and highly conserved throughout unicellular, such as eukaryotes and prokaryotes, as well as multicellular organisms, including plants, fungi and animals (Hanna and Abouheif, 2022). The induction of PCD is an essential response to a multitude of intracellular and extracellular stimuli, for example cellular stress factors, paracrine signals or infections with pathogens (Golstein and Kroemer, 2005). Additionally, PCD is implicated in inflammation and the development and integrity of multicellular organisms, which involves morphogenesis, tissue homeostasis and reproduction as well as regulation of cell cycle and differentiation (Ameisen, 2002). Overall, the execution of PCD serves as pivotal measure to eliminate potential harmful or infected cells and to sacrifice superfluous cells, which benefits the whole organism (Bedoui et al., 2020). During infection with intracellular pathogens, it contributes to an effective immune response to restrict the survival and replication of bacteria, viruses and parasites within host cells. Dysregulation of PCD is the reason for numerous pathologies, for instance developmental disorders, immunodeficiency, autoimmune diseases and cancer (Gibellini and Moro, 2021).

Various PCD pathways, including pyroptosis, necroptosis and apoptosis, and their main functions and outcomes have been described within the last decades (Green, 2019). Furthermore, a myriad of cellular sensors, initiator and effector molecules associated with the initiation, regulation and execution of cell death have been characterised (Galluzzi et al., 2018). However, there is emerging evidence that distinct types of PCD are more connected with each other than initially thought and we just begin to unravel their flexibility, redundancy and wide-ranging functions (Green, 2019).

Caspases are the key players within the network of different PCD pathways and critical for inflammation and the activation of cell death. Besides that, caspases are involved in non-cell death processes, such as cell proliferation and differentiation (Nakajima and Kuranaga, 2017, Nhan et al., 2006, Schwerk and Schulze-Osthoff, 2003, Shalini et al., 2015). While the primary functions of most members of the caspase family are well described, the roles of some caspases, including caspases-2 and -12, remain enigmatic (Bouchier-Hayes and Green, 2012, Fava et al., 2012, Krumschnabel et al., 2009, Lamkanfi et al., 2004, Vakifahmetoglu-Norberg and Zhivotovsky, 2010). Additionally, potential redundant or compensatory roles of caspases are incompletely understood.

Due to the critical role of PCD in health and disease (Gibellini and Moro, 2021) it is of utmost importance to investigate the complex network of cell death by disentangling the overlapping functions of cell death initiator and executioner molecules as well as the interconnectivity between different pathways and their regulation systematically. This will provide crucial knowledge for the identification of drug targets and the development of novel treatment strategies for various diseases.

We addressed this by infecting novel genetically modified mouse models and macrophage cell lines deficient for different combinations of PCD effector molecules with the intracellular bacterial model pathogen *S.* Typhimurium. The deletion of key molecules of single pathways or a combination of multiple pathways allowed us to analyse the relative importance and of pyroptosis, necroptosis and apoptosis during the control and clearance of intracellular infections. Furthermore, we characterised the roles of several caspases and mechanisms of cell death induction during infections with *S.* Typhimurium.

6.2 The redundancy and interconnectivity of different programmed cell death pathways

Interestingly, we observed a remarkable level of redundancy in the regulation of the PCD pathways pyroptosis, necroptosis and extrinsic apoptosis during infection with *S.* Typhimurium. Mice deficient for individual or combinations of two different of these pathways controlled the infection and restricted bacterial replication without major impairments. Furthermore, our findings indicate that caspase-1/-11 mediated pyroptosis and caspase-8 mediated apoptosis are essential for control of *S.* Typhimurium infections, while necroptosis itself is not critical (**Figure 6.1**). The combined loss of pyroptosis, necroptosis and caspase-8 mediated apoptosis led to uncontrolled replication of *S.* Typhimurium and the death of infected mice. This indicates that additional types of PCD, including intrinsic apoptosis, as well as other immune responses, such as the production of ROS, are not sufficient to control *S.* Typhimurium infections. Additional experiments revealed that the control of *S.* Typhimurium breaks down if mice can no longer purge intracellular bacteria from the immune cell compartment by the induction of PCD. These findings reiterate the importance of phagocytes as replicative niche for intracellular pathogens. The extensive crosstalk between pyroptosis, necroptosis and apoptosis following infection was also reported by other studies, which infected macrophages with influenza A virus, vesicular stomatitis virus, *Listeria monocytogenes* and *S.* Typhimurium (Christgen et al., 2020, Place et al., 2021).

To further disentangle the observed complexity of the PCD network and the response of macrophages to infections with *S.* Typhimurium we established sensitive *in vitro* approaches to measure cell death kinetics in real-time and determine the intracellular bacterial burden of infected cells. Our results indicate that caspases-1 and -8 are the key players in the control and clearance of *S.* Typhimurium infections. Pyroptosis deficient macrophages induce caspase-8 mediated apoptotic cell death with delayed kinetics following infection with *S.* Typhimurium, indicating that apoptosis functions as default backup

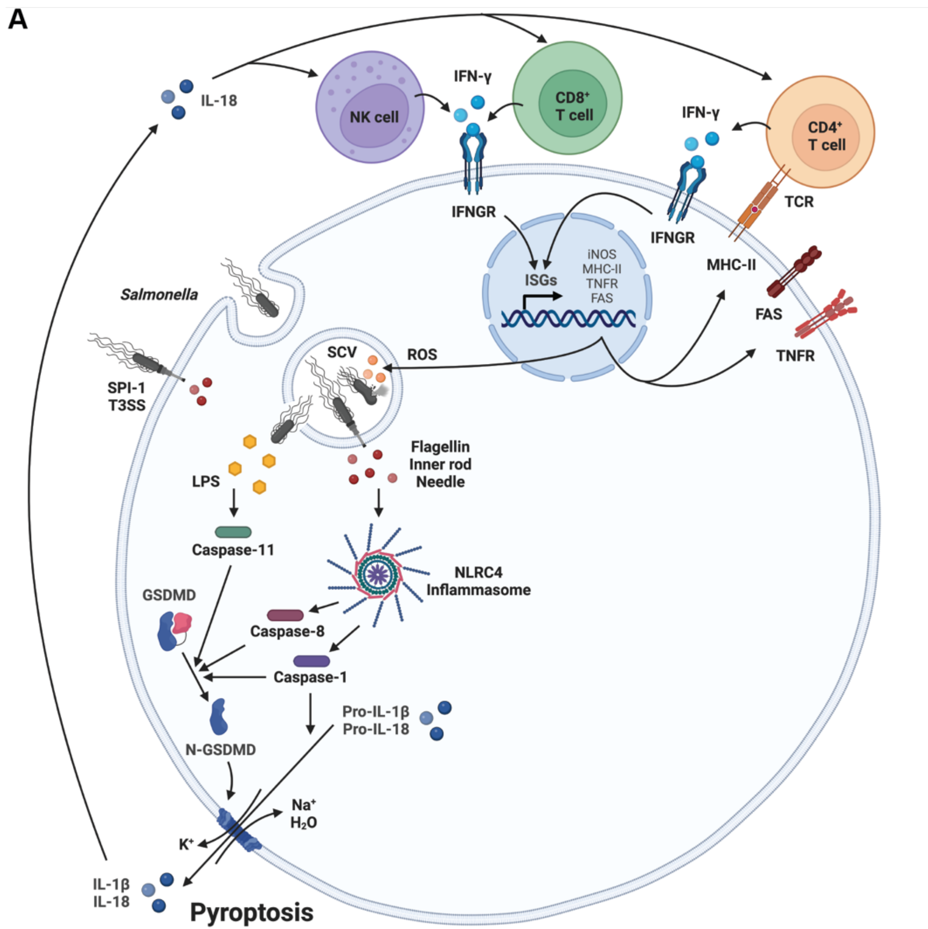
mechanism in the absence of pyroptosis. This confirms previous reports, which have reported that caspase-8 can be activated by inflammasomes in certain scenarios (Antonopoulos et al., 2015, Lee et al., 2018, Mascarenhas et al., 2017, Pierini et al., 2012, Rauch et al., 2017, Sagulenko et al., 2013, Schneider et al., 2017, Van Opdenbosch et al., 2017). Pyroptosis, necroptosis and apoptosis deficient macrophages were resistant to *S.* Typhimurium induced cell death, resulting in intracellular bacterial survival and replication. This resistance to *Salmonella*-induced cell death is most likely the explanation for the severely elevated bacterial numbers in liver and spleen of infected PCD-deficient mice. Similar *in vitro* results were observed for infections with influenza A virus, vesicular stomatitis virus and *Listeria monocytogenes* (Christgen et al., 2020). Additional findings of us revealed the high interconnectivity between pyroptosis and apoptosis by describing novel functions for caspases-1 and -8 (Doerflinger et al., 2020). The pyroptotic caspase-1 can induce apoptosis by activation of apoptotic effector caspases-3, -7 and -9 independently of caspases-8 and BID (Doerflinger et al., 2020). Furthermore, we showed that caspases-1 and -8 act as cell death executioners themselves in the absence of all known cell death effector molecules (Doerflinger et al., 2020). This implies that caspases-1 and -8 possess more functions than previous described or the existence of additional downstream executioners activated by caspases-1 and/or -8 during *S.* Typhimurium infection.

Taken together, our findings contribute to the increasing evidence that different PCD pathways are highly connected at multiple levels and flexibly induced during intracellular infections. Rather than being autonomous processes, it is most likely that pyroptosis, necroptosis and apoptosis are part of one big cell death system that responds with different kinetics to a multitude of challenges dependent on the cell type and the stimuli (Bedoui et al., 2020, Place et al., 2021, Samir et al., 2020).

The plasticity between PCD pathways is most prominent during the immune response to infection, while apoptosis is the most important PCD mechanism of

organisms to impede the development of cancer (Bedoui et al., 2020). This is most likely because of the co-evolution of the host immune system with invading pathogens. The multiple in-build fail-safe processes protect the organism from various intracellular bacterial pathogens, which have developed extensive strategies and an array of effector molecules that interfere with their detection by the host and the subsequent induction and execution of PCD (Wemyss and Pearson, 2019). The remarkable flexibility and interconnectivity within the PCD network allow the immune system to compensate for the loss or evasion of pyroptosis with the activation of the apoptotic pathway (Bedoui et al., 2020). This provides multiple ways to restrict intracellular pathogen survival and replication, induce an inflammatory response and ensure pathogen control and clearance. However, the crosstalk between pyroptosis, necroptosis and apoptosis with caspases-1 and -8 as master regulators was also observed during embryogenesis and development in recent reports (Newton et al., 2019a, Newton et al., 2019b).

S. Typhimurium is a master manipulator of host cells and interferes with PCD on multiple levels (Wemyss and Pearson, 2019). Following cellular invasion, *S.* Typhimurium modifies its surface intensively and downregulates various PAMPs that trigger inflammasome assembly and subsequent induction of PCD and inflammation. For example, *S.* Typhimurium switches the expression from SPI-1 effectors, which are important for motility and invasion, to the expression SPI-2 effectors, that are essential for establishing the SCV and intracellular survival. Our findings confirmed previous reports showing that flagellin or SPI-1 T3SS deficient *S.* Typhimurium strains evade inflammasome activation and pyroptosis induction (Jesenberger et al., 2000, Lightfield et al., 2008, Miao et al., 2006). We also observed that these mutant bacteria prevent the induction of inflammasome induced apoptosis via caspase-8 *in vitro*. However, we demonstrated that mice infected with flagellin or SPI-1 T3SS deficient *S.* Typhimurium strains are able to control the infection without any impairments. This implies the involvement of additional mechanisms of PCD initiation during *S.* Typhimurium infection within the more complex *in vivo* system.


A
IL-18
NK cell
IFN-γ
CD8+ T cell
IFN-γ
CD4+ T cell
IFNGR
TCR
IFNGR
ISGs
iNOS
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TNFR
FAS
MHC-II
FAS
TNFR
Salmonella
SCV
ROS
SPI-1 T3SS
LPS
Flagellin
Inner rod
Needle
Caspase-11
NLRC4 Inflammasome
GSDMD
Caspase-8
Caspase-1
Pro-IL-1β
Pro-IL-18
N-GSDMD
Na+
H2O
K+
IL-1β
IL-18
Pyroptosis

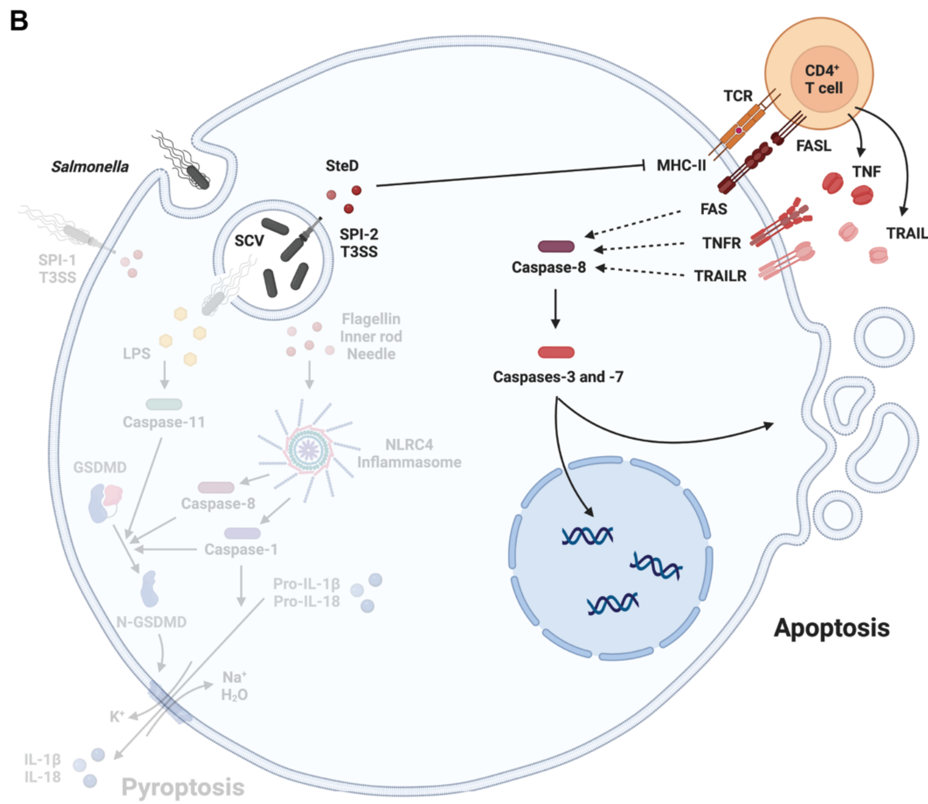
B
CD4+ T cell
TCR
Salmonella
SteD
MHC-II
FASL
TNF
SCV
SPI-2 T3SS
FAS
TRAIL
SPI-1 T3SS
TNFR
Caspase-8
TRAILR
LPS
Flagellin
Inner rod
Needle
Caspases-3 and -7
Caspase-11
NLRC4 Inflammasome
GSDMD
Caspase-8
Caspase-1
Pro-IL-1β
Pro-IL-18
N-GSDMD
Apoptosis
Na+
H2O
K+
IL-1β
IL-18
Pyroptosis

Figure 6.1: Cellular and molecular immune effector mechanisms and their regulation during *Salmonella* Typhimurium infection.
(A) The detection of cytosolic *Salmonella* proteins, including flagellin and structural components of the *Salmonella* pathogenicity island (SPI)-1 encoded type III secretion system (T3SS), initiates the assembly of inflammasomes. Following recruitment and activation within the NLR family CARD domain-containing 4 (NLRC4) inflammasome, caspases-1 and -8 cleave gasdermin D (GSDMD). The N-terminal GSDMD fragment translocates to the plasma membrane and oligomerises to form pores resulting in pyroptotic cell death. In contrast, caspase-11 directly senses cytosolic *Salmonella* and is activated by lipopolysaccharide (LPS). Activated caspase-11 can also cleave GSDMD, thus providing an alternative path towards cell lysis. Pyroptotic cell death results in the release of pro-inflammatory cytokines, such as interleukin 1 beta (IL-1β) and interleukin 18 (IL-18), which are cleaved into their bioactive forms by caspase-1. IL-18 can induce the release of interferon gamma (IFN-γ) by activated natural killer (NK) cells, CD8$^+$ T cells and CD4$^+$ T cells. By acting on phagocytes, IFN-γ stimulates antimicrobial capacities, such as reactive oxygen species (ROS) production, antigen presentation and expression of death receptors. (B) *Salmonella* limits inflammasome activation and pyroptosis by downregulation of SPI-1 and flagellin, and interferes with major histocompatibility complex (MHC) class II antigen presentation. While caspase-8-dependent apoptosis can compensate for the lack of pyroptotic cell death, it is not clear what triggers caspase-8 under these circumstances. Considering that the SPI-2 effector protein SteD depletes mature MHC class II molecules from the surface of infected cells, that effector CD4$^+$ T cells can express Fas ligand (FasL) and that IFN-γ enhances the expression of MHC class II molecules and the receptor for FasL, it is possible that the caspase-8-dependent switch from pyroptosis to apoptosis is orchestrated by *Salmonella*-specific CD4$^+$ T cells. Simplified overview of cellular and molecular requirements for cell death induction during *Salmonella* infection depicted (Figure published in Engel et al, 2023).

6.3 The involvement of $CD4^+$ T cells and IFN-γ in the induction of caspase-8 mediated apoptosis

While the importance of $CD4^+$ T cells and IFN-γ for the control and clearance of intracellular infection is well described (Hess et al., 1996, Kupz et al., 2014), their involvement in the induction of PCD is not completely understood. Therefore, we investigated the potential role of $CD4^+$ T cells and IFN-γ in orchestrating the flexible use of extrinsic apoptosis as effective immune response to limit intracellular bacterial survival and replication in this thesis. In conclusion, our findings suggest that cytotoxic $CD4^+$ T cells are crucial for the clearance of *S.* Typhimurium infections (**Figure 6.1**). The induction of caspase-8 mediated extrinsic apoptosis via Fas/FasL by $CD4^+$ T cells seems to be important to eliminate intracellular bacteria that evade pyroptosis, while TNF-α and TRAIL play only minor roles. Our findings provide evidence that *S.* Typhimurium effectively inhibits this process by interfering with the surface levels of MHC-II. Previous reports have indicated that *S.* Typhimurium interferes with antigen presentation and the activation and proliferation of T cells (Bayer-Santos et al., 2016, Jackson et al., 2013, Lapaque et al., 2009, McLaughlin and van der Velden, 2016, Mitchell et al., 2004). The *S.* Typhimurium effector SteD promotes ubiquitination and subsequent depletion of surface MHC-II by binding to the host E3 ubiquitin ligase MARCH8 (Bayer-Santos et al., 2016, McLaughlin and van der Velden, 2016). MHC-II molecules accumulate in intracellular vesicles in *S.* Typhimurium infected cells instead (Jackson et al., 2013, Mitchell et al., 2004). It is intriguing to speculate if the purpose of SteD-mediated manipulation of surface MHC-II by *S.* Typhimurium is to prevent the induction of apoptosis of infected cells rather than interfering with T cell priming.

Furthermore, our data implies that IFN-γ secreted during infection counteracts this evasion strategy of *S.* Typhimurium by sensitising macrophages to extrinsic apoptosis induction, most likely by promoting the expression of MHC-II and the

death receptors Fas and TNFR1. Previous studies also reported that IFN-γ upregulates the expression of MHC-II on macrophages (Kalupahana et al., 2005).

Additional reports indicate that IFN-γ enhances Fas/FasL expression and increases Fas-mediated apoptosis in tumour cell lines (Xu et al., 1998, Zheng et al., 2002) and microglia (Badie et al., 2000). Furthermore, it was shown that IFN-γ regulates the expression of FasL in human CD4$^+$ T cells and thereby controls their anti-mycobacterial cytotoxic functions (Boselli et al., 2007). Collectively, these findings support the hypothesis that IFN-γ influences the induction of PCD by promoting the recognition of infected cells as well as the expression of cell death effectors and receptors.

Strikingly, similar findings were obtained for *Mycobacterium tuberculosis* infections of human macrophages *in vitro* (Bocchino et al., 2005). Monocyte-derived macrophages were sensitive to FasL induced apoptosis, but exposure to TNF-α did not induce cell death (Oddo et al., 1998). The induction of apoptosis in infected macrophages was associated with a reduced viability of intracellular *Mycobacterium tuberculosis* bacteria (Oddo et al., 1998). However, infected cells showed a reduced susceptibility to FasL induced apoptosis, most likely because of the interference of *Mycobacterium tuberculosis* with the expression levels of Fas (Oddo et al., 1998). Additionally, it was reported that *Mycobacterium tuberculosis* infection reduced the expression of costimulatory molecules and MHC-II on macrophages (Gercken et al., 1994, Saha et al., 1994). These strategies allow *Mycobacterium tuberculosis* to dampen immune responses through interfering with antigen presentation and cytotoxic CD4$^+$ T cell induced killing (Bocchino et al., 2005).

The importance of the Fas/FasL signalling pathway for apoptosis induction and infection control is further reflected in the fact that several pathogens, besides *Mycobacterium tuberculosis*, directly target this cell death mediated host defence mechanism (Caulfield and Lathem, 2014). While *Yersinia pestis* cleaves FasL found on the surface of effector cells (Caulfield et al., 2014), enteropathogenic

Escherichia coli modifies FADD within target cells to prevent Fas mediated apoptosis (Li et al., 2013, Pearson et al., 2013).

6.4 Outlook

We have yet to confirm our conclusions regarding the role of $CD4^+$ T cells and Fas/FasL in the induction of extrinsic apoptosis presented in this thesis. The cellular and molecular requirements for cell death induction following *S.* Typhimurium infection could be addressed by generating mixed bone marrow chimeric mice in which the induction of extrinsic apoptosis is only possible in MHC-II deficient immune cells. Infection of such mice will provide detailed insights in the involvement and importance of cytotoxic $CD4^+$ T cells for extrinsic apoptosis initiation during intracellular infections. Besides that, the potential impairments in the control of *S.* Typhimurium infections within mice lacking FasL would provide further validation of our results. This approach will extend on the obtained *in vitro* findings and could manifest the importance of FasL or reveal the redundancy within the TNF superfamily for the induction of extrinsic apoptosis *in vivo*. The analysis of RNA sequencing datasets of activated $CD4^+$ T cells following infection with *S.* Typhimurium could reveal more details regarding their cytotoxic profile, including their potential to secret IFN-γ as well as members of the TNF superfamily. Additionally, imaging of liver and spleen sections of WT and PCD deficient mice following infection with *S.* Typhimurium could help to identify which monocyte and/or macrophage subsets are predominantly infected with *S.* Typhimurium and serve as intracellular niche for bacterial survival and replication. Furthermore, the co-localisation and potential interactions of cytotoxic $CD4^+$ T cells with infected cells could be uncovered. A flow cytometric approach could determine if infected monocyte/macrophage subsets express and upregulate members of the TNF receptor superfamily and MHC-II during infection and the potential of *S.* Typhimurium to interfere with $CD4^+$ T cell induced cell death.

The use of antibiotics has revolutionised modern medicine and drastically decreased the mortality and morbidity of bacterial infectious diseases. However, the development of antibiotic resistances and the formation of bacterial persisters were discovered shortly after the onset of the use of antibiotics. Antibiotics are one of the most prescribed drugs worldwide and their use constantly increases. The mis- and overuse of antibiotics accelerates the development of resistances and led to several multi-drug resistant bacteria. Furthermore, the discovery of new antibiotics to fight bacterial infections has significantly slowed down.

Persisters are a subpopulation that change to a dormant state following cell invasion. They are non- or slow-growing bacteria that are tolerant to antibiotics. The phenomenon of bacterial persisters is a major cause of antibiotic treatment failure and the development of chronic and recurrent infections, which results in the further spread of the pathogen. In this context it is noteworthy, that the reason for the widespread observation that infection of cells, even with high MOI, does not result in the death of every infected cell is still not completely understood. If this is due to persister formation, inhibition of PCD, reprogramming of the infected cell by the pathogen, natural cellular resistance to infection or a combination of different factors needs further investigation (Green, 2019). Interestingly, *S.* Typhimurium was described as ideal model organism to study antibiotic persistence (Newson et al., 2022). It is intriguing to speculate that the PCD of infected cells could serve as effective mechanism to eliminate bacterial persisters, which are not responding to antimicrobial therapy.

One potential therapeutic approach to treat antibiotic resistant bacteria is to enhance essential immune responses important for limiting bacterial replication instead of targeting the invading pathogen itself. The pharmacological induction of PCD during intracellular infection with *Legionella* by repurposing approved anti-cancer drugs was reported to be efficient to limit bacterial replication and prevent lethal lung infections *in vivo* (Speir et al., 2016). BH3 mimetics are small molecules that mimic members of the pro-apoptotic BH3-only protein family, which inhibit pro-survival BCL-2 proteins and thereby promote the induction of

intrinsic apoptosis. We investigated the effects of BH3 mimetics on our *S.* Typhimurium infection model. Preliminary results suggest that administering BH3-mimetics limits bacterial replication in *S.* Typhimurium infected mice (unpublished data). However, due to the unspecific nature of cell death induction and off target effects on uninfected immune cell subsets important for bacterial control, an optimal dose and administration regiment is crucial. Another class of PCD inducing drugs that have been shown to enhance apoptosis and promote *Mycobacterium tuberculosis* clearance *in vivo* are IAP antagonists (Stutz et al., 2021). IAP antagonists are clinical-stage compounds that block the caspase inhibitory function of XIAP. Pharmacological induction of apoptosis were also shown to kill latent HIV-infected $CD4^{+}$ T cells (Campbell et al., 2018, Cummins et al., 2016) as well as hepatitis B virus (Ebert et al., 2015) and *Plasmodium* parasite infected hepatocytes (Ebert et al., 2020), which enhances clearance of the virus *in vivo*. Therefore, PCD inducing agents serve as promising complementary strategy or alternative to conventional antimicrobial and antiviral drugs (Naderer, 2017).

6.5 Concluding remarks

In summary, we used sophisticated multiple knockout *in vivo* and *in vitro* models to disentangle the highly flexible and interconnected network of PCD pathways, which is of fundamental importance for health and disease. Our findings contributed to answer long-lasting questions within the field of cell death research regarding the relative importance and regulation of pyroptosis, necroptosis and apoptosis and unravelled novel functions for several PCD initiator and effector molecules and mechanisms for cell death induction following intracellular infection. This will be beneficial for the identification of drug targets and novel therapeutic approaches against intracellular infections.

Furthermore, the generated multiple knockout mouse and macrophage cell lines as well as established *in vitro* assays are useful tools for various approaches to investigate unresolved scientific questions in the fields of developmental biology, immunology and infection, host – pathogen interactions and cancer.

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