

**DENDRITIC CELLS MEDIATE PROTECTIVE IMMUNITY
AGAINST SALMONELLA TYPHIMURIUM BY
REGULATING ANTIGEN PRESENTATION,
INFLAMMATION AND CELL DEATH**

by

Rajen Patel, M.Sc
Bachelor of Science, McGill University, 2008
Master's of Science, University of Ottawa, 2010

THESIS SUBMITTED TO THE FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE

DOCTORATE IN PHILOSOPHY

DEGREE IN THE
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

Department of Microbiology and Immunology
Faculty of Medicine
University of Ottawa

© Rajen Patel, Ottawa, Canada, 2016

ABSTRACT

Salmonella enterica serovar Typhimurium (ST) is an intracellular bacterium that resides within the phagosome of infected cells. ST is the causative agent of gastroenteritis in humans and typhoid like disease in mice. ST infects epithelial cells and phagocytic cells such as dendritic cells (DCs), which are immune sentinels that have been regarded as the most critical antigen-presenting cell (APC). I evaluated the role of CD8 α DCs, a subset of DCs capable of antigen presentation of phagosomal pathogens to activate CD8⁺ T cells. Furthermore, I assessed the role of key cytokines such as the group of classical anti-viral cytokines known as Interferon-I (IFN-I), on licensing CD8⁺ T cells. Interestingly, IFN-I signalling was necessary for production of inflammatory cytokines and induction of cell death, which activated CD8⁺ T cells and clearance of ST. Lastly, I examined the role of key cell death pathways in innate immune protection against ST. In particular, I addressed how signalling pathways in necroptosis and pyroptosis are critical for the production of IL-1 and IL-18 which mediate immune protection against ST. Determining the mechanisms of which DCs engage innate and adaptive immune responses against phagosomal bacteria is the central question of my study and is addressed by examining critical roles of DC function, inflammation and cell death.

I dedicate my PhD thesis to my wife, my brother and my parents.

ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to all of those who have helped made my graduate studies possible throughout the years. First, I would like to thank all the members of the Sad laboratory, both past and present, for their input and support throughout my time in the lab. I would also like to thank the members of Lakshmi Krishnan's lab for thoughtful discussion and input during our lab meetings at the NRC. My work would not be possible without the guidance of my thesis advisory committee members Seung-Hwan Lee, Jonathan Angel and Lionel Filion. I would like to thank Fanny Tzelepis for her mentorship and guidance during my initial years as a PhD student. Thanks to Kim Kwangsin, Renu Dudani and Komal Gurnani for providing technical support throughout my studies. In addition, I would like to express my sincere gratitude to Julie Joseph, Kathryn Wright, Norah Alturki and Virja Mehta for critical analysis of my thesis. I would like to thank FRSQ and OGS for providing funding for my PhD fellowships. I would also like to thank all the collaborators for providing reagents and mice. Most importantly, I would like to thank Dr. Subash Sad for giving me the opportunity to pursue scientific research in his laboratory and for encouraging me to go beyond my limits, generate meaningful data and in the process, learn about my capabilities as a scientist.

TABLE OF CONTENTS

Abstract	ii
Dedication.....	iii
Acknowledgements	iv
Table of Contents	v
List of Figures	viii
List of Tables	xii
List of Abbreviations	xiii
1: Introduction	1
1.1 Immune Response	1
1.2 Innate Immunity.....	2
1.3 Antigen Processing Pathways.....	5
1.4 Adaptive Immune Response	10
1.5 Antigen Presenting Cells	14
1.6 Monocytes and Macrophages	17
1.7 Dendritic Cells	19
1.8 Batf3-Dependent DCs	22
1.9 Mononuclear Phagocyte Ontogeny.....	27
1.10 <i>Salmonella</i> Pathogenesis.....	32
1.11 Innate Immune Response against <i>Salmonella</i>	36
1.12 Interferons	38
1.13 Programmed Cell Death.....	41
1.14 Apoptosis.....	45
1.15 Necroptosis	47
1.16 Regulation of Apoptosis and Necroptosis	49
1.17 Pyroptosis and Inflammation	53
1.18 Rationale	56
1.19 Purpose	57
1.20 Hypothesis.....	58
1.21 Objectives.....	58
1.21.1 Aim1	61
1.21.2 Aim2	61
1.21.3 Aim3	61
2: Materials and Methods.....	62
2.1 Mouse Strains	62
2.2 Bacteria and Infections.....	69
2.3 Antibodies and Flow Cytometry.....	70

2.4	Inhibitors and other Reagents	71
2.5	CFSE Staining.....	71
2.6	ELISPOT	72
2.7	<i>In-vivo</i> CTL	73
2.8	Adoptive Transfer	74
2.9	Isolation of DCs and other cells.....	74
2.10	Bone Marrow Derived Macrophages and DCs.....	75
2.11	<i>In-vitro</i> Infection Assay	79
2.12	LPS Treatment	80
2.13	Cell Death Assays	80
2.14	Cytokine and Chemokine Quantification	81
2.15	Histology.....	82
2.16	Western Blot.....	82
2.17	Statistical Analysis.....	83
3:	Results	84
3.1	CHAPTER 1: IFN-I signalling is critical to mount a CD8 ⁺ T cell response against ST	84
3.1.1	CD8 ⁺ T cells mediate immune protection against ST	84
3.1.2	ST infected DCs fail to mediate early antigen presentation.....	91
3.1.3	Poor CD8 ⁺ T cell response in IFNAR1-deficient mice following ST infection	94
3.1.4	Critical role of IFNAR signalling in CD8 ⁺ T cell priming is independent of antigen location within the infected cell	105
3.1.5	Impact of IFNAR on CD8 ⁺ T cell priming is pathogen dependent	108
3.1.6	Migratory DCs lacking IFNAR expression are defective in inflammatory responses and cell death	115
3.2	CHAPTER 2: Batf3-dependent DCs are critical for development of CD8 ⁺ T cell responses against <i>Salmonella</i> Typhimurium regardless of the location of antigen.....	133
3.2.1	CD8 α DCs display increased uptake of ST antigen	133
3.2.2	Poor CD8 ⁺ T cell response in Batf3-deficient mice.....	136
3.2.3	CD8 ⁺ T cell response in Batf3-deficient mice correlates with the number of CD8 α DCs	147
3.2.4	Batf3-dependent DCs are critical to mount a CD8 ⁺ T cell response against ST in the draining lymph nodes	152
3.2.5	Batf3-dependent DCs are a critical source of inflammatory cytokines and chemokines during ST infection	152
3.3	CHAPTER 3: Synergism of Caspase1/11 and RipK3 signalling pathways in control of intracellular bacteria	162
3.3.1	Early antigen presentation is necessary to reveal the protective role of inflammatory cell death pathways	162
3.3.2	Expression a functional <i>Nramp1</i> gene reveals the critical role of Caspase1/11- and RipK3-signalling during ST infection	171
3.3.3	Disruption of both Caspase1/11 and RipK3 results in accelerated host fatality	174
3.3.4	Synergistic impact of Caspase1/11 and RipK3 in inflammasome activation and cell-death.....	180

3.3.5	Caspase-1, Caspase-8 and RipK3 mediate processing of IL-1 cytokines.....	194
3.3.6	Regulation of inflammasome activation by RipK3 kinase activity.....	202
3.3.7	Regulation of inflammasome activation by cellular inhibitors of apoptosis proteins	208
3.3.8	Administration of IL-18 reduces the ST bacterial burden in Caspase1/11 and RipK3-deficient mice	213
4:	Discussion	221
5:	Copyright Permission	250
6:	Reference List.....	270

LIST OF FIGURES

Figure 1: Antigen presentation	8
Figure 2: Mononuclear phagocytes	16
Figure 3: Transcriptional regulation of dendritic cells	25
Figure 4: Mononuclear phagocyte development	30
Figure 5: <i>Salmonella</i> Typhimurium	35
Figure 6: Pathways of cell death	44
Figure 7: Modulation of CD8 ⁺ T cell response by antigen location	60
Figure 8: <i>In-vitro</i> cultures of dendritic cells	78
Figure 9: CD4 ⁺ and CD8 ⁺ T cells mediate control of chronic ST in resistant mice.....	86
Figure 10: CD8 ⁺ T cells mediate immune control of ST in Nramp1 expressing mice	90
Figure 11: Rapid antigen presentation is not induced by DCs infected with ST	93
Figure 12: Cross-presentation during ST infection is partially dependent on IFN-I during inflammatory conditions induced by ST.....	97
Figure 13: Priming of CD8 ⁺ T cells against OVA protein in the context of an ST infection requires IFN-I signalling	99
Figure 14: IFNAR is critical for localized infection of ST.....	102
Figure 15: IFNAR is not required for systemic ST infection.....	104
Figure 16: ST-OVA and ST-OVA-C are both virulent recombinant strains of ST	107
Figure 17: Recombinant strain of ST that deposits OVA into the cytoplasm of infected cells induces rapid CD8 ⁺ T cell response	110
Figure 18: IFNAR1-deficient mice exhibit delayed CD8 ⁺ T cell kinetics against localized infection with ST-OVA-C	112
Figure 19: IFNAR signalling has no impact on CD8 ⁺ T cell response against systemic ST-OVA-C	114

Figure 20: IFNAR signalling is not required for CD8 ⁺ T cell responses against localized LM infection	117
Figure 21: IFNAR has no impact on CD8 ⁺ T cell response against systemic LM.....	119
Figure 22: CD103i DCs require IFNAR to induce ST mediated cell death	122
Figure 23: CD103i DCs require IFNAR signalling for optimal cytokine and chemokine production following ST infection	124
Figure 24: GMCSF-DCs require IFNAR signalling for cytokine and chemokine production following ST infection.....	127
Figure 25: Reduced infiltration of migratory and resident DCs in the draining lymph nodes of IFNAR1-deficient mice following ST-OVA-C infection	129
Figure 26: Visual Abstract I: IFNAR signalling is required for protective CD8 ⁺ T cell response against ST	132
Figure 27: ST is selectively acquired by Batf3-dependent DCs	135
Figure 28: Delayed CD8 ⁺ T cell kinetics against ST infection in Batf3 deficient mice	138
Figure 29: Batf3 promotes CD8 ⁺ T cell response against chronic ST infection	141
Figure 30: Batf3 is important for early CD8 ⁺ T cell priming against ST when antigen is translocated to the cytoplasm	144
Figure 31: Delayed proliferation of OT-1 CD8 ⁺ T cells following ST-OVA-C infection in Batf3-deficient hosts.....	146
Figure 32: CD8 α DCs re-emerge in Batf3-deficient mice following ST infection	149
Figure 33: Batf3 impacts host survival against recombinant ST-OVA-C	151
Figure 34: Batf3 promotes early CD8 ⁺ T cell priming in a localized ST infection model.....	154
Figure 35: Reduced production of inflammatory cytokines and chemokines in Batf3-deficient DCs following ST infection	157
Figure 36: Impaired cytokine production in Batf3-deficient bone marrow derived cells following ST infection	159
Figure 37: Visual Abstract II: Batf3-dependent DCs are required for immune protection against ST independent of the location of immunogenic antigen	161
Figure 38: Caspase1/11 signalling provides minimal impact on immune protection against phagosomal ST infection	164

Figure 39: Engaging early antigen presentation reveals the protective role of Caspase1/11 signalling against ST infection.....	167
Figure 40: RipK3 plays a protective role only when antigen presentation is engaged early during infection	170
Figure 41: Expression of Nramp1 enhancing the survival of B6 mice to reveals the critical role of Caspase-1/11 and RipK3 signalling with phagosomal ST	173
Figure 42: Synergism of Caspase-1/11 and RipK3 signalling during ST infection	176
Figure 43: Diminished immune cell subsets following infection of RipK3, Caspase1/11 and double deficient mice with ST	179
Figure 44: Caspase1/11 and RipK3 are critical to mediate ST induced cell-death and inflammasome activation following infection of macrophages and DCs.....	182
Figure 45: Induction of cytokine production in Caspase1/11- and RipK3-deficient macrophages and DCs following ST infection.....	185
Figure 46: Induction of cytokine production in Caspase1/11- and RipK3-deficient macrophages and DCs following LPS treatment.....	187
Figure 47: <i>In-vivo</i> LPS treatment reveals deficiency in cytokine production in Caspase1/11- and RipK3-deficient mice	189
Figure 48: Caspase1/11- and RipK3-deficient mice exhibit attenuation of IL-18 response following ST infection.....	191
Figure 49: Caspase1/11- and RipK3-deficient macrophages and DCs are able to promote NF- κ B (p65) and MAPK (p38) signalling following ST infection	193
Figure 50: Caspase-1/11, Caspase-8 and RipK3 are critical signalling pathways to mediate IL-1 β production in macrophages and DCs in response to ST infection	196
Figure 51: Macrophages deficient in Caspase-1/11 and RipK3 signalling pathways express normal level of proteins critical for inflammation.....	199
Figure 52: DCs deficient in Caspase-1/11 and RipK3 signalling pathways express normal level of proteins critical for inflammation.....	201
Figure 53: In the absence of Caspase-1/11, Caspase-8 and RipK3 are critical signalling pathways to mediate IL-1 β production against flagellin in DCs.....	204
Figure 54: Inhibition of RipK3 kinase activity potentiates IL-1 β cytokine production in DCs.....	207

Figure 55: cIAP1/2 negatively regulates IL-1 β cytokine production in DCs.....	210
Figure 56: In the absence of Caspase1/11, cIAP1/2 expression is increased	212
Figure 57: IL-18 plays a protective role in Caspase-1/11- and RipK3-deficient mice following ST infection.....	215
Figure 58: Visual Abstract III-A: Caspase-1/11, Caspase-8 and RipK3 promote comprehensive inflammasome activation against ST	218
Figure 59: Visual Abstract III-B: Impact of Caspases and RipK3 on inflammasome activation and host survival against ST.....	220
Figure 60: Thesis Overview.....	249

LIST OF TABLES

Table 1: Mouse models.....	64
Table 2: PCR primers	67

LIST OF ABBREVIATIONS

ACVS	Animal care and veterinary services
AP-1	Activator protein-1
APAF	Apoptosis-activating factor
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
Batf	Basic leucine zipper transcription factor ATF-like
BCG	Bacillus Calmette-Guérin
BDCA	Blood dendritic cell antigen
BHI	Brain heart infusion
Bir	Birinapant
BMDC	Bone marrow derived dendritic cells
BMM	Bone marrow derived macrophage
Bst-2	Bone marrow stromal cell antigen-2
CARD	Caspase activation and recruitment domain
Casp	Caspase
CD	Cluster of Differentiation
CD103i DC	CD103 induced bone marrow derived DCs
cDC	Conventional dendritic cell

CDP	Common DC progenitors
cIAP	Cellular inhibitor of apoptosis
CMP	Common myeloid progenitors
CpG ODN	CpG oligodeoxynucleotide
CrmA	Cytokine response modifier A
CSF-1R	Colony stimulating factor 1 receptor
CTL	Cytotoxic T lymphocyte
CYLD	Cylindromatosis
DAMP	Danger associated molecular patterns
Dbn	Dabrafenib
DC	Dendritic cell
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
ECL	Enhanced chemiluminescent
ELISPOT	Enzyme-linked immunospot assays
ER	Endoplasmic reticulum
ERAP1	Endoplasmic reticulum-associated amino-peptidase 1
FADD	Fas-associated protein with death domain

FLT3L	FMS-related tyrosine kinase 3 ligand
FLT3L-DC	Bone marrow derived DCs grown with FLT3L
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMCSF-DC	Bone marrow derived DCs grown with GMCSF
H&E	Hematoxylin and eosin
HBSS	Hank's balanced salt solution
HMGB1	High mobility group protein B1
HRP	Horse radish peroxidase
HSCs	Hematopoietic stem cells
IFN	Interferon
IFNAR1	Interferon alpha receptor 1
IL	Interleukins
IRAP	Insulin-responsive amino peptidase
IRF	Interferon-regulatory factor
JAK1	Janus kinase 1
KC	keratinocyte-derived cytokine
LCMV	Lymphocytic choriomeningitis virus
LCN2	Lipocalin-2
LM	<i>Listeria monocytogenes</i>
LPS	lipopolysaccharide
M cells	Microfold cells
M-CSF	Macrophage colony stimulating factor

MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MCMV	Mouse Cytomegalovirus
MCP-1	Monocyte chemoattractant protein-1
MDPs	Macrophage-DC progenitors
MHC	Major histocompatibility complex
MIG	Monocyte induced by gamma interferon
MIP	Macrophage inflammatory protein
MLKL	Mixed lineage kinase domain-like
MMPs	Metallophilic macrophages
MoDCs	Monocyte-derived DCs
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
Mϕ	Macrophage
NADPH	Nicotinamide adenine dinucleotide phosphate
NAIP5	Neuronal apoptosis inhibitor protein 5
Nec-1	Necrostatin-1
NF-κB	Nuclear factor- κ B
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain receptors
NLRC4	NLR family, CARD domain containing 4
NLRP3	NLR family, pyrin domain containing 3

NOD	Nucleotide-binding oligomerization domain receptors
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid dendritic cell
polyI:C	Polyinosinic:polycytidylic
PR3	Proteinase 3
PRR	Pattern recognition receptors
R8%	RPMI + 8% Fetal calf serum
RANTES	Regulated on activation normal T cell expressed and secreted
RHIM	Receptor interacting protein homotypic interaction motif
RipK	Receptor interacting protein kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Siglec	Sialic acid-binding immunoglobulin-type lectin
SMAC	Second mitochondria-derived activator of caspases
SPI	<i>Salmonella</i> pathogenicity island
ss	Single stranded

ST	<i>Salmonella enterica serovar Typhimurium</i>
ST-OVA	<i>Salmonella Typhimurium</i> expressing OVA
ST-OVA-C	<i>Salmonella Typhimurium</i> expressing YopE-OVA
STAT	Signal transducer and activator of transcription
T3SS	Type three secretion system
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TIR	Toll/IL-1R homologous region
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
TRADD	TNF receptor type 1 associated death domain
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter inducing interferon- β
TYK2	Tyrosine kinase 2
USP	Ubiquitin-specific peptidases
UTP	Uridine triphosphate
VSV	Vesicular stomatitis virus
zVAD	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone
β-ME	β -Mercaptoethanol
β2m	β 2-Microglobulin

1: INTRODUCTION

1.1 Immune Response

The immune system has evolved to include a hierarchy of white blood cells to provide protection against foreign substances within a given host. An intricate network of immune sentinel cells orchestrates comprehensive control of foreign invaders by mediating effector functions for pathogen destruction and secretion of soluble factors to recruit or activate additional immune cells. The primary barrier to infection commences with mechanical (movement of mucus), chemical (antibacterial peptides) and microbiological (microbiome) defences present in a given host (1). Following the breach of these barriers, sentinel immune cells are recruited to the site of infection to capture and destroy any invading pathogen, leading to the activation of the innate and adaptive immune responses for comprehensive pathogen control.

The innate immune system provides rapid defence against foreign invaders. Innate immune cells express germ-line encoded receptors that respond promptly to a broad range of pathogens by mediating anti-microbial effector functions and promoting an influx of additional immune cells to the site of infection. This is in contrast to the adaptive immune system that fine-tunes an

immune response against a particular antigen (or more precisely specific part of an immunogenic protein known as the epitope) through clonal selection and gene rearrangement (2, 3). Consequently, the adaptive immune response is more robust and highly specific towards an invading pathogen. Furthermore, innate immune sentinels, such as dendritic cells (DCs) have been identified as a link between the innate and adaptive immune responses. DCs have the capacity to capture antigen through phagocytosis (engulfment), process the antigen in the phagosome (a vesicle which contains engulfed pathogens from the environment) and prime the adaptive immune response (4). The interaction between the innate and adaptive immune system can be manipulated by pathogens to dampen the immune response allowing the pathogen to persist within the host.

1.2 Innate Immunity

The innate immune response is the first line of defence against foreign invaders. Innate immune cells recognize invading pathogens by engagement of pathogen recognition receptors (PRRs) that recognize a broad range of pathogen associated molecular patterns (PAMPs) (5, 6). These receptors are expressed in various compartments of the cell where pathogens can be detected, such as the plasma membrane, endosome or cytosol (7). PAMPs such as bacterial lipopolysaccharide (LPS) or viral double stranded ribonucleic acid (dsRNA) are uniquely expressed by pathogens and are not associated with the host, making them ideal targets for immune activation (5). PRRs such as Toll-like receptors (TLRs) are expressed on the plasma membrane (or endosomes), which allow them to detect extracellular pathogens (8). In contrast, PRRs expressed in the

cytosol, such as NOD (nucleotide-binding oligomerization domain receptors)-like receptors (NLRs) recognize intracellular pathogens (8). Engagement of PRRs leads to multiple signalling cascades, resulting in the expression of soluble proteins such as cytokines and chemokines to direct immune function and recruitment of immune cells to the site of infection respectively (8). Specialized sentinel cells such as macrophages have an enhanced capacity for proteolysis following recognition by TLRs and NLRs, and thus represent a critical population for early innate immune control of pathogenic burden. Furthermore, immune activation through PRR stimulation leads to critical antimicrobial functions such as expression of reactive oxygen species (ROS), acidification of the phagosome or secretion of antimicrobial peptides to eliminate invading pathogens (9). Pathogens that reside in the cytoplasm of infected cells can be detected by NLRs, which subsequently forms a multi-protein complex known as the inflammasome (10). Activation of the inflammasome results in the processing of pro-inflammatory cytokines and induction of inflammatory cell death (10).

Innate immune control of invading pathogens can be dictated by PRRs expressed on the plasma membrane or in the cytosol of immune sentinel cells. TLRs are PRRs that survey the extracellular environment for pathogenic motifs. TLR-1/2, -4, -5 and -6 are expressed on the surface of the cell and recognize lipoproteins, LPS, flagellin and zymosan, respectively (8). In contrast, TLR-3, -7/8, and -9 are expressed within the endosome or phagosome, an environment where extracellular pathogens are captured and degraded, thus allowing for detection of dsRNA, viral single stranded ribonucleic acid (ssRNA) and bacterial

CpG ODN (CpG oligodeoxynucleotides) respectively (8). TLRs generally utilize two main signalling pathways: (i) TIR (Toll/IL-1R homologous region)-domain-containing adapter inducing interferon- β (TRIF) dependent signalling pathway that leads to immune cell activation and (ii) myeloid differentiation primary response gene 88 (MyD88) dependent signalling resulting in inflammatory cytokines (11). Both signalling pathways in concert provide comprehensive inflammatory responses against pathogens. Inflammatory cytokines promote inflammation, which allows immune cells to activate effector functions and promote recruitment of immune cells to the site of infection (through chemotaxis and blood vessel dilation). TLR3 operates exclusively through the TRIF pathway, while all other TLRs can engage the MyD88 pathway (11). Interestingly, only TLR4 is capable of operating through both MyD88 and TRIF signalling pathways (11). Detection of pathogens through engagement of PRRs is a major mechanism of immune surveillance.

Many intracellular pathogens reside and proliferate within the cytoplasm of infected cells, evading detection through TLR activation. In this case, PRRs such as NLRs are engaged to detect cellular stress or perturbations that originate from the cytoplasmic compartment of the cell (10). While TLRs detect mostly PAMPs, NLRs are capable of detecting ligands of both pathogenic and host origin, indicating their importance in detecting general stress within the cell. For instance, NLR family caspase activation and recruitment domain (CARD) containing 4 (NLRC4) receptor recognizes bacterial products such as flagellin (12) or rod proteins present in the cytoplasm (13). In comparison, NLR family

pyrin domain containing 3 (NLRP3) receptor can detect intracellular contents of either pathogen (bacterial, fungal, viral) or host origin (external adenosine triphosphate (ATP), uric acid, crystals) (10). Both NLRs lead to activation of a multimeric complex called the inflammasome leading to production of pro-inflammatory cytokines such as Interleukin (IL)-1 α , IL-1 β and IL-18 and induction of inflammatory cell death through pyroptosis (10).

Cellular stress induced by pathogens or injury can lead to cell necrosis, which results in the release of danger associated molecular patterns (DAMPs) normally sequestered within healthy cells (14). Indeed, detection of DAMPs released by infected cells has been proposed to be an important mechanism of immune surveillance (14). Common DAMPs released by damaged and infected cells include extracellular adenosine tri-phosphate (ATP), high-mobility group box 1 (HMGB1), IL-1 α , IL-1 β and IL-18. Comprehensive detection of both PAMPs and DAMPs of various origins allows the innate immune system to respond to invading microbes.

1.3 Antigen Processing Pathways

Innate immune cells provide a rapid but broad defence against invading pathogens. Furthermore, specialized innate immune cells known as antigen-presenting cells (APCs) process and present pathogen-derived antigen during the early stages of infection to shape a specific adaptive immune response to promote pathogen control. The process of acquiring pathogenic antigen through phagocytosis, proteolytic degradation of the acquired antigenic and loading the

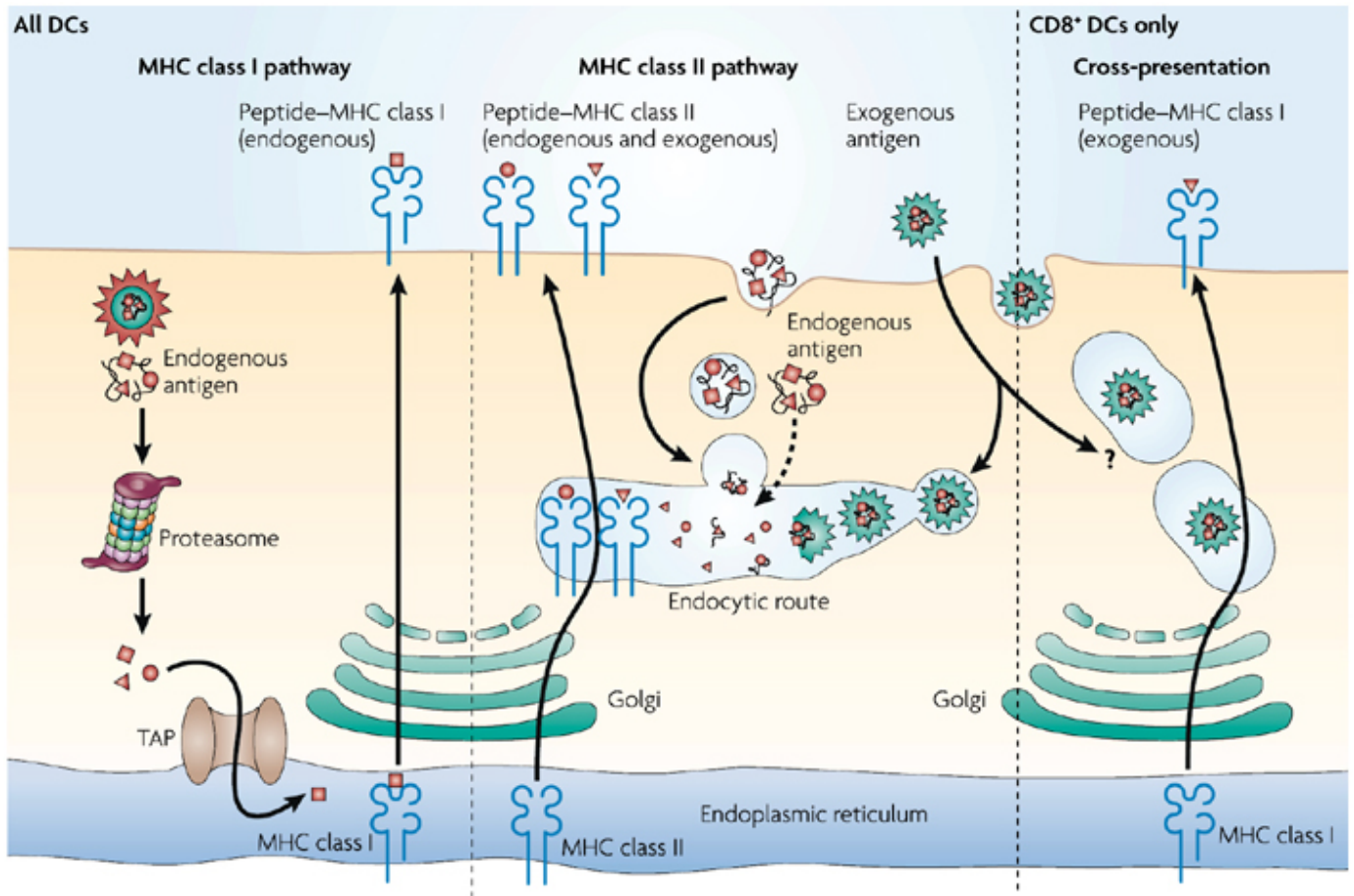
generated peptides onto major histocompatibility complex (MHC) molecules on the surface of the cell leads to antigen presentation (15). Interestingly, DCs have been shown to be the most critical population of APCs that have the capacity to activate T cells *in-vivo* (16-18).

A critical component to antigen presentation is the loading of peptides generated from pathogens onto MHC molecules. T cell receptors (TCRs) expressed on cluster of differentiation (CD)8⁺ and CD4⁺ T cell populations bind to peptides processed and presented onto MHC class-I and MHC class-II molecules respectively (19). CD8⁺ T cells are primed by peptides presented on MHC class-I molecules, which consists of three domains ($\alpha1\alpha2\alpha3$) in complex with a $\beta2$ -microglobulin ($\beta2m$) molecule. MHC class-I molecules are expressed on all nucleated cells and allow recognition of the intracellular compartment (19). In contrast, antigens acquired from the extracellular environment are captured into vesicles (phagosomes) and loaded onto MHC class-II, which consists of two trans-membrane glycoproteins ($\alpha1\alpha2$ and $\beta1\beta2$), and are recognized by CD4⁺ T cells (19). MHC class-II expression is exclusive to APCs such as DCs and activated macrophages. Antigens from extracellular pathogens are acquired through phagocytosis, a function that is generally restricted to APCs, and the peptides generated by proteases at low pH in the phagosome are presented onto MHC class-II molecules to prime CD4⁺ T cells (**Fig. 1**) (19). In contrast, antigens that originate from intracellular pathogens that are localized in the cytosol of infected cells are readily degraded by proteasomes, which is common to all cell

Figure 1: Antigen presentation

All nucleated cells are capable of presenting endogenous antigen onto MHC-I molecules while only APCs have the capacity to promote MHC class-II presentation of exogenous antigens. Antigen located in the cytoplasm can be processed by the proteasome and loaded onto MHC class-I molecules to activate CD8⁺ T cells. In contrast, antigen can also be captured from the extracellular environment through a process called phagocytosis and loaded onto MHC class-II molecules. In certain cases, a CD8⁺ T cell response is required for immune protection against phagosomal antigen. In such cases, specialized CD8 α DCs are capable of presenting phagosomal antigen onto MHC class-I molecules through a process called cross-presentation.

Figure as originally published in Villadangos JA et al. (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol 7:7.



types as all nucleated cells constantly present self and non-self antigens (**Fig. 1**) (19).

While MHC class-I and –II molecules are specific for CD8⁺ and CD4⁺ T cell recognition respectively, CD8⁺ T cells can also be activated, in some instances, by an alternate pathway, referred to as cross-presentation, wherein phagosomal antigens are presented to CD8⁺ T cells in the context of MHC class-I molecules (**Fig. 1**) (20-22). This can be critical in cases where pathogens that do not infect APCs require a CD8⁺ T cell response (23). Phagosomal antigen has been described to be processed for cross-presentation through two distinct pathways: the cytosolic (proteasome dependent) and the vacuolar (endosomal protease dependent) cross-presentation pathways (22).

Cross-presentation is a mechanism of presenting exogenous antigens on MHC class-I molecules to allow for priming of CD8⁺ T cells, which is often an essential component for comprehensive control of an invading pathogen (23). Following internalization of exogenous antigen in the phagosome, two main pathways have been described for cross-presentation: the cytosolic pathway and the vacuolar pathway (24). The dominant pathway utilized by cross-presenting APCs may depend on numerous factors, such as the cell type acquiring the antigen, the nature of the antigen (cellular debris or soluble protein) and the inflammatory conditions which the APCs were exposed to when acquiring the antigen (LPS, cytokines) (24).

The cytosolic pathway requires transfer of antigen from the lumen of the phagosome to the proteasome for degradation and processing (25). Following

antigen processing by the proteasome, the peptides generated are either transferred to the endoplasmic reticulum (ER) by a mechanism dependent on transporter associated with antigen processing (TAP) proteins to shuttle peptides from the cytosol to the ER for MHC class-I loading or may also enter the endocytic compartment to be loaded onto an MHC class-I molecule (21, 24). In contrast, the vacuolar pathway is resistant to proteosomal inhibitors as it depends on lysosomal proteolysis of antigen captured in the phagosome, by proteases such as cathepsins (26). A study of particular interest administered exogenous cytochrome c into mice, which resulted in cell death of various DC populations (27). Cytochrome c is a protein generally sequestered in the mitochondria, which is released following pro-apoptotic signals and induces caspase mediated cell death. DCs are able to acquire cytochrome c through phagocytosis and allow access of cytochrome c to the cytoplasm as these DCs are engaging the cytosolic cross-presentation pathway resulting in cell death (27). Thus, evidence shows that the cytosolic pathway appears to be the more relevant pathway for cross-presentation *in-vivo* (28).

1.4 Adaptive Immune Response

The adaptive immune response consists of both cell mediated and humoral immunity. Cell mediated immunity is driven by cytotoxic CD8⁺ T cells, which recognize and kill abnormal or infected cells. CD8⁺ T cells express perforin (which polymerize to form pores in the plasma membrane) and granzymes (which induce apoptosis) to lyse target cells (29). Humoral immunity consists of B cells, which secrete high affinity neutralizing antibody against extracellular

pathogens and toxins (29). CD4⁺ T cells can promote either cell mediated or humoral immunity depending on the characteristics of the invading pathogen based on stimulation by either a CD4⁺ helper T (T_H)1 or T_H2 response (29). Intracellular pathogens generally promote T_H1 cells, which secrete interferon (IFN)- γ and tumor necrosis factor (TNF)- α to promote macrophage anti-microbial functions and pathogen control (29). In the absence of inflammation during infection, CD4⁺ T_H1 help can be required to promote CD8⁺ T cell priming through cytokine production and CD40 signalling (30, 31). In contrast, CD4⁺ T_H2 cells promote B cell activation by secreting B cell growth factors such as IL-4, IL-5 and IL-13 (29). Comprehensively, CD4⁺ T cells can help dictate the most suitable immune response depending on the type of pathogen (intracellular or extracellular) and the inflammatory conditions, which the pathogen induces.

CD4⁺ and CD8⁺ T cells are characterized by their unique ability for clonal expansion of antigen specific T cells, which can result in a 1000-10000 fold increase following DC priming (29). Depending on the nature of the pathogen, sometimes only a CD4⁺ T cell response is required for eradication of the pathogen, and in other cases both CD4⁺ and CD8⁺ T cells are required. Following resolution of the pathogen, CD4⁺ and CD8⁺ T cell response undergoes rapid contraction, following programmed cell death to limit the number of antigen specific T cells present in the host (29). Following control of the pathogen, contraction of the response results in elimination of ~ 90% of the activated cells, and survival of less than 10% cells as memory T cells, which are a population of antigen specific T cells maintained for long periods of time and play a critical

protective role during re-exposure to the pathogen (29). Contraction is also a mechanism that helps maintain homeostasis by creating more room for new naïve T cells that have to respond to other infections.

Primed CD4⁺ and CD8⁺ T cells that survive contraction persist in the host as effector memory cells in non-lymphoid organs and central memory cells in lymphoid organs (32). Effector memory T cells are capable of rapidly mediating effector T cell functions and are capable of migrating to inflamed tissues. In contrast, central memory T cells have diminished effector functions but are capable of proliferating and differentiating into effector cells to mediate secondary immune responses (32). Memory T cells sustain homeostatic cell proliferation by maintaining low levels of cell proliferation and cell death to retain a small numbers of memory T cells prior to re-exposure to a pathogen (32). Interestingly, memory T cells are able to provide long term protective immunity by lowering their threshold of activation to induce a rapid and robust response upon re-exposure (29). Changes in gene expression and surface protein expression helps maintain the transition from naïve to memory T cells. For instance, memory CD4⁺ and CD8⁺ T cells undergo reprogramming that is mediated by changes in chromatin structure and transcription factor expression (33). Furthermore, changes in expression of cell adhesion molecules and receptors for chemotaxis allow functional attributes unique to memory T cells.

APCs such as DCs can dictate the type of adaptive immune response elicited against a particular pathogen by regulating antigen presentation. Naïve T cells are able to respond to pathogens by recognizing antigens presented onto

APCs, which induces clonal expansion of naïve T cells that express a specific TCR for the MHC-peptide complex presented (19). APCs also express co-stimulatory molecules, which encourage activation of T cells upon binding of TCR to the MHC-peptide complex, allowing for activation and clonal expansion of the T cells (34). This induces T cell differentiation that results in the development of effector T cells, which expand to cytotoxic CD8⁺ T cells (CTLs) and CD4⁺ T cells (29).

Efficient activation and proliferation of naïve T cells by DCs requires three types of signals: (i) TCR binding to MHC-peptide complex along with co-receptors CD4 or CD8, (ii) signals from co-stimulatory molecules expressed by DCs and T cells (i.e. CD28 and CD80) and (iii) cytokine signalling (34). Signal 1 is critical for T cell activation, mediated by TCR recognition of the MHC-peptide complex. Interestingly, APC-CD4⁺ T cell expansion can occur as quickly as 6 hours, in comparison APC-CD8⁺ T cell expansion can start in 2 hours following priming (35-41). Signal 2 is mediated by a array of co-stimulatory molecules such as CD28-CD80 and CD40-CD154, which vary in signal strength depending on the type of infection and the type of T cell engaged (42-44). The presence of more abundant antigen generally results in a stronger CD8⁺ T cell response (45). However, this does not always lead to enhanced resolution of infection, as infected cells are sometimes not recognized by the CD8⁺ T cells due to virulence mechanisms employed by some pathogens (46). Furthermore, prolonged antigen levels due to chronic infection leads to slower proliferation, loss of function (termed exhaustion) and deletion (47, 48), which can result following lymphocytic

choriomeningitis virus (LCMV) infection. Signal 3 is provided by various APCs such as DCs, which secrete cytokines such as IL-12 and IFN- α to promote proliferation and effector function of primed T cells (49).

1.5 Antigen Presenting Cells

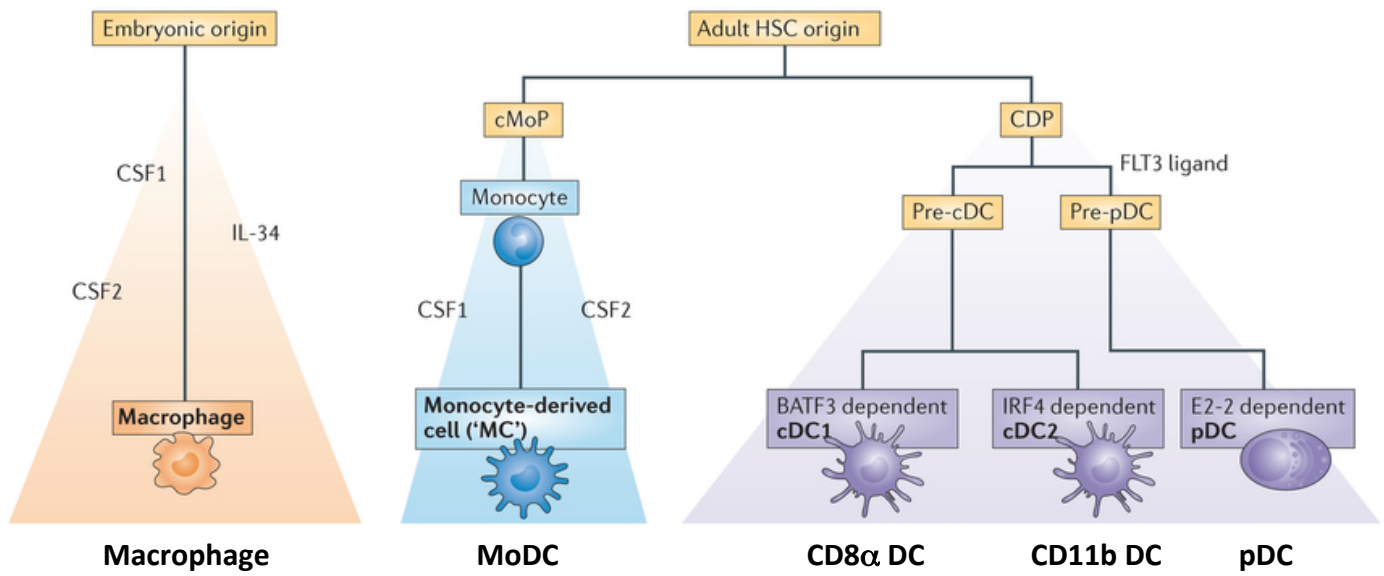
Mononuclear phagocytes is a collective term for myeloid cells that includes monocytes, macrophages and DCs (**Fig. 2**). These cells share various cell markers that were once thought to be unique to each subset. Consequently, the use of ontogeny and immune function is now preferred to differentiate between these various cell types (50). For instance, initially it was suggested that both macrophages and DCs were relevant APCs capable of activating an adaptive immune response within a given host. However, further analysis using ontogeny and immune function suggested that various DC subsets are most efficient at priming T cells *in-vivo* (50-52).

Following phagocytosis of an extracellular antigen, DCs engage limited antigen degradation to preserve the integrity of the peptides generated (53, 54). Thus DCs are capable of maintaining environments with higher pH levels in the phagosome to maintain antigen integrity for both MHC class-I loading leading to effective priming of the adaptive immune response (53, 55). In contrast, macrophages are inherently more proteolytic as they express more proteases and maintain a lower pH in the phagosome (55). Furthermore, LPS induces maturation of DCs that leads to enhanced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, promoting its antigen presentation capabilities (56). This phenotype occurs exclusively in DCs and does not occur in

Figure 2: Mononuclear phagocytes

The mononuclear phagocytes consist of mainly four groups of cells, monocytes, macrophages, conventional DCs and pDCs. Macrophages can be derived from either circulating monocytes or seeded cells from embryonic origin during birth. Macrophage development is dependent on M-CSF (CSF1) and GM-CSF (CSF2) and in some cases IL-34, while DC development is dependent on FLT3L. Common DC progenitors (CDPs) can give rise to Batf3-dependent CD8 α DCs, IRF4-dependent CD11b DCs and E2-2-dependent plasmacytoid DCs (pDCs).

Figure as originally published in Guilliams M et al. (2014) Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol 14:8 doi: 10.1038/nri3712.



other mononuclear phagocytes such as macrophages (55). Maintaining such a harsh environment in the phagosome results in pathogen destruction yielding peptides that are not optimal for MHC presentation (57).

In addition to enhanced capabilities for antigen presentation, DCs have additional characteristics that make them superior at priming T cells (51). DCs have also been shown to have superior capabilities to cross-present phagosomal antigens to CD8⁺ T cells (19). Expression of various receptors such as mannose receptors and DNGR-1 (CLEC9A), increases their ability for cross-presentation of soluble proteins and necrotic cells respectively (58-60). In addition, DCs express high levels of co-stimulatory molecules such as CD40, CD80 and CD86 that provide signal 2, which is critical for priming of naïve T cells (34). Furthermore, DCs have the capacity to migrate to draining lymph nodes, while macrophages generally remain localized to the site of infection (61). Chemokine signalling and even physical stress can induce rapid movement of DCs, underlining the enhanced ability of DCs to migrate to draining lymph nodes where naïve T cells are located (61). Ablation in the DC population results in impairment of the adaptive immune response (18). Collectively, research has shown that DCs are the most capable APC for antigen presentation and priming of T cells *in-vivo*.

1.6 Monocytes and Macrophages

Monocytes and macrophages are critical for innate immune control of invading pathogens (62). For instance, monocytes are critical for maintaining inflammation during microbial challenge, whereas macrophages maintain tissue homeostasis and resolve pathogen induced inflammation (63-65). Originally, it

was widely accepted that monocytes were circulating precursors of macrophages, which differentiate to replenish macrophage populations (62). However, recent insights have revealed that adult tissue macrophages can be seeded before birth into tissues from embryonic precursors from the yolk sac and fetal liver (64, 66-68). Furthermore, macrophages can self renew the resident populations independent of monocyte precursors (67, 69). Indeed, new insights that challenged the central dogma of monocyte to macrophage differentiation have had major implications for therapeutics against various pathogens (**Fig. 2**) (64).

Macrophages are known as vacuolar cells that excel at pathogen control and uptake of cellular debris from lysed cells. These cells have been associated with expression of the cell surface marker F4/80 (64). However, expression of F4/80 can be variable depending on the tissue origin (64). Macrophages can arise from differentiated monocytes. However, monocytes themselves can also mediate pathogen control within inflammatory environments. Indeed both monocytes and macrophages represent distinct cell types critical for immune protection. Monocytes can be characterized into two distinct subsets: patrolling monocytes (Ly6C^{low} CX₃CR1^{hi} CCR2^{low}) and inflammatory monocytes (Ly6C^{hi} CX₃CR1^{low} CCR2^{hi}) (64). Interestingly, monocytes originate from the bone marrow and have the potential to differentiate into both macrophages or DCs (62). Following infection, the majority of DCs migrating to the site of infection are derived from monocytes called monocyte derived DCs (MoDCs), which serve as a critical source of “emergency DCs” which are required at the site of infection

following encounter with a pathogen (70). MoDCs are classically known to upregulate maturation associated molecules and functions upon encounter with a pathogen or stress signal, which allows the DCs to migrate to the draining lymph nodes and initiate an adaptive immune response (70).

Tissue resident macrophages are considered to promote anti-inflammatory functions and homeostasis along with maintaining tissue specific functions and tissue repair (64, 71). Tissue resident macrophages such as liver resident Kupffer cells acquire specific functions to aid in the development and function of the liver. For instance, Kupffer cells phagocytose and break down red blood cells in the liver sinusoids (72). However, following inflammatory stimulation macrophages become critical for host defence (65). Macrophages control bacterial burden early on during infection (63). Upon engagement of PRRs, macrophages quickly engulf extracellular pathogens and utilize ROS and nitric oxide species (NOS) to degrade foreign invaders (63). Macrophages have high capacity for catabolic activity, and thus represent a critical population for innate immune control of pathogen burden.

1.7 Dendritic Cells

DCs were first described as “large stellate” cells with distinct dendrite extensions isolated from adherent splenocytes (16). DCs were restricted to the integrin marker CD11c, which was used to isolate and characterize these cells. However, their autonomy from the closely related macrophage was initially in question as both myeloid cells were shown to have similar functional characteristics (50). Studies revealed that DCs are a highly unique and

specialized subset of myeloid cells that are capable of specialized antigen presentation, migration, and are developed from a subset of progenitor cells, all of which are distinct from macrophage (51). DCs are the most critical subset of antigen-presenting cells capable of orchestrating an appropriate T cell response against a pathogen. Without the presence of a DC population, the adaptive immune response is severely compromised (51).

DCs represent a heterogeneous population consisting of various resident and migratory cells with unique functions and ontogeny (52). DCs are generally classified as either conventional DCs (cDCs) or plasmacytoid DCs (pDCs) (52). Interestingly, pDCs have a very distinct spherical morphology; an appearance that closely resembles plasma B cells. pDCs are specialized DCs that respond to viral infection by secreting large amounts of the anti-viral cytokine known as IFN-I (52). Expression of pDC markers such as B220, sialic acid-binding immunoglobulin-like lectin (Siglec)-H and bone marrow stromal cell antigen 2 (Bst2) are commonly used to discriminate pDCs from cDCs (73, 74). Despite being classified as DCs, pDCs have not been shown to be efficient at mediating antigen presentation, partly due to the high turnover rate of MHC on the surface of the cell (75). Thus, the primary characterized function of pDCs has been shown to be cytokine production following viral infection.

cDCs are specialized cells with high capacity to process captured antigens and pathogens for presentation to T cells (52). They are relatively short-lived with a half-life of about 3-5 days (76). As a consequence, they are constantly replenished by progenitors originating in the bone marrow following stimulation

with the growth factor FMS-related tyrosine kinase 3 ligand (FLT3L) (**Fig. 2**) (77-79). cDCs are typically classified as tissue resident or lymphoid resident DCs (52). Tissue resident DCs include subsets such as Langerhans cells in the epidermal layer of the skin and intestinal CD103⁺CD11b⁺ DCs which survey the gut environment (80, 81). Lymphoid resident DCs include CD8 α DCs and CD11b⁺ DCs in lymphoid organs such as the spleen and draining lymph nodes (82, 83). These DCs are capable of acquiring antigen that is present in lymphoid organs and process them for antigen presentation (52).

DCs were first characterized in the epidermal layer of the skin (51). Langerhans cells are a special subset of DCs, characterized by the expression of langerin (CD207) and Birbeck granules and reside in the epidermal layer of the skin (84, 85). Langerhans cells survey the environment for any invading pathogens. Once a pathogen has been detected in the epidermal layer, Langerhans cells engulf the pathogen through phagocytosis (86). The antigens captured in the phagosome are processed through proteolysis and presented onto MHC molecules for antigen presentation (51). This process also allows the DC to transition from an immature patrolling DC to a mature DC capable of priming a T cell response (51, 87). Maturation also allows expression of co-stimulatory molecules (ie. CD40, CD80, CD86), which are critical to prime and activate a CD4⁺ and CD8⁺ T cell response towards the invading pathogen (51). Immature DCs are known to be patrolling cells. In this state, they express low levels of co-stimulatory molecules, MHC-II molecules and are highly phagocytic, and constantly sampling the environment for self and non-self antigens (51, 87).

Upon recognition of a pathogen (ie. TLR or NLR activation), DCs transition to a mature phenotype, which down-regulates phagocytic capabilities, and up-regulates MHC class-II and co-stimulatory molecules (51, 87). Furthermore, DC activation allows expression of key cytokine receptors such as CCR7 to allow proper migration to the T cell zones of the draining lymph nodes to allow interactions with naïve CD4⁺ and CD8⁺ T cells (88).

Langerhans cells have been the DC subset that has helped shape the paradigm for DC activation and T cell priming (89). Despite their morphological similarities to other DC subsets, Langerhans cells may be more accurately characterized as macrophages. This clarification has been based on the findings that macrophage colony stimulating factor (M-CSF) receptor expression is critical for Langerhans cell development (90). Furthermore, Langerhans cell populations are normal in granulocyte-macrophage colony-stimulating factor (GM-CSF) and FLT3L-deficient mice, the latter of which lack all DC subsets (85). Indeed, DCs are innate immune cells, which excel at antigen capture and pathogen control, which in turn leads to DC maturation, migration and antigen presentation. However, under inflammatory conditions, or niche tissue environments, additional immune populations have evolved the capacity for antigen presentation and T cell priming (91).

1.8 Batf3-Dependent DCs

Cross-presentation is an alternative pathway of antigen presentation for exogenous antigen onto MHC-I molecules. Only a few subsets of DCs are capable of engaging cross-presentation and allowing phagosomal antigens to be

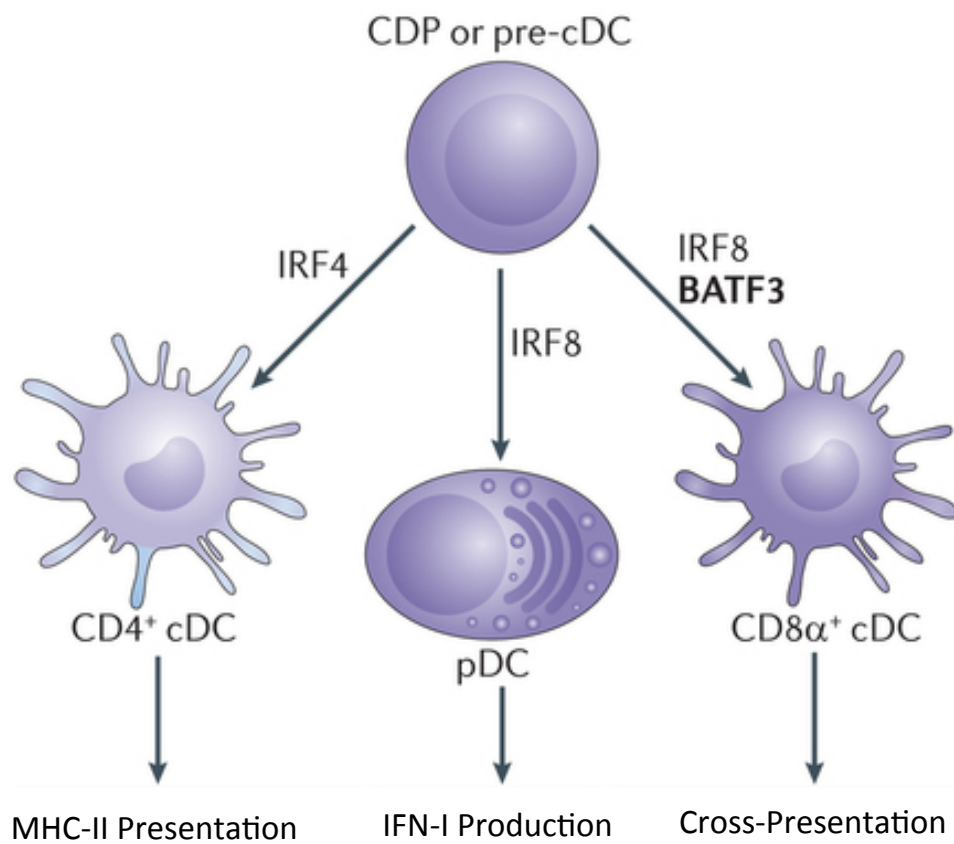
processed onto an MHC class-I molecule to prime cytotoxic CD8⁺ T cells (82, 83, 92). Cross-presentation is especially critical in cases where pathogens have evolved to avoid infecting DCs, as a mechanism of immune evasion (23). Furthermore, cross-presentation is critical in situations where antigen is available as cellular debris following cell death, such as with certain non-haematopoietic cancers (23). DCs (93-95), macrophages (96, 97), B cells (98) and neutrophils (99) have some capacity to present exogenous antigen to MHC class-I molecules *in-vitro*. However, DCs are the only relevant population to cross-present *in-vivo* (95). For instance, the transgenic DTR-CD11c mouse strain depletes DCs upon diphtheria toxin administration in drinking water, which results in abrogation of CD8⁺ T cell response to cell associated antigen (18). Furthermore, DCs have been shown to be up to 1000 times more capable of cross-presentation than macrophages (55, 100).

Lymphoid resident CD8 α DCs and tissue resident CD103⁺ DCs are subsets that specialize in cross-presentation (**Fig. 3**) (82, 101, 102). CD8 α DCs expresses specialized processing machinery capable of cross-presentation (82). In comparison, another subset called CD11b DCs (also referred to as CD4⁺ DCs) process and present antigen mainly through direct-presentation (52, 83, 103). At steady state, CD8 α DCs are strategically located in the T cell zone and marginal zone of lymphoid organs such as the spleen, which allows them to present captured antigens to naïve CD4⁺ and CD8⁺ T cells (104, 105). CD103⁺ DCs exhibit similar transcriptional signatures to CD8 α DCs (106). However, during steady state, they are located in peripheral tissues. CD103⁺ DCs are critical for

Figure 3: Transcriptional regulation of dendritic cells

Different DC subsets can develop from common DC progenitors (CDPs) in the bone marrow. Batf3 is a critical transcription factor required for the development of CD8 α DCs (and CD103⁺ DCs in peripheral lymphoid tissues). Importantly, deficiency in Batf3 does not impact other DC subsets such as pDCs and CD11b DCs (also known as CD4⁺ DCs). It is important to note that both CD8 α and CD103⁺ DCs are also dependent on interferon regulatory factor IRF-8; which also impacts pDC development. Thus Batf3-deficient mice and not IRF-8-deficient mice were used as an ideal model to study the impact of CD8 α DCs during various immune responses.

Figure as originally published in Murphy TL et al. (2013) Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks. Nat Rev Immunol 13:7 doi: 10.1038/nri3470.



priming CD8⁺ T cells either as a source of antigen (107) or for mediating cross-presentation of phagosomal antigens following activation and migration to the draining lymph nodes (106). CD8 α DCs and CD103⁺ DCs express many PRR such as NLRs, TLRs and produce cytokines such as IL-12 and IFNs, which in certain cases are critical early on during an infection (108-111). CD8 α and CD103⁺ DCs also express DNGR-1 (CLEC9A) receptor, which endows these DCs to pick up exposed F-actin from necrotic cells for cross-presentation (59, 112). Cells that have engaged death following stress or infection can represent a critical source of antigen for priming the adaptive immune response against pathogens or tumor cells (94, 113, 114). All DC subsets to date are capable of acquiring fluorescent ovalbumin (OVA) *in-vivo*, yet only CD8 α DCs are able to cross-present the antigen (82). CD8 α DCs are a subset that is more capable of cross-presenting soluble antigen (115), viral antigen (116, 117), bacterial antigen (92) and apoptotic debris (118). Furthermore, CD8 α DCs are needed for cross-presentation of antigens in the spleen (115, 118) and lymph nodes (119). Other DC subsets have been shown to induce cross-presentation under specific inflammatory conditions, such as MoDCs, as a possible source of emergency cross-presenting DCs (120, 121). Nevertheless, CD8 α DCs have been shown to be the most critical cross-presenting APC *in-vivo* (**Fig. 3**) (101).

Basic leucine zipper transcription factor ATF-like (Batf)-3 is an activator protein-1 (AP-1) transcriptional factor critical for the development of CD8 α DCs (**Fig. 3**) (52, 101). Batf3-deficient mice lack the splenic CD8 α DC population while maintaining proper development of other DC subsets (101). Batf3-deficient

mice have been shown to have diminished cross-presentation capabilities *in-vivo* (101). Furthermore, Batf3-dependent DCs have been shown to be critical for a CD8⁺ T cell response following implantation of solid tumor models (101) or pathogenic challenge (101, 109, 122). Pathogens such as *Listeria monocytogenes* (LM) require Batf3-dependent DCs to transit from the blood to specific areas of the spleen to induce productive infection (122). Furthermore, CD8 α DCs are a critical early source of IL-12 following *Toxoplasma gondii* infection. Infection of Batf3-deficient mice with *Toxoplasma gondii* leads to early host fatality (109). Indeed Batf3-dependent DCs mediate innate and adaptive immunity against various pathogens.

1.9 Mononuclear Phagocyte Ontogeny

Monocytes, macrophages and DCs develop from progenitor cells in the bone marrow from hematopoietic stem cells (50, 123-125). However, tissue resident macrophages can also develop from seeded cells in tissues capable of self-renewal (66). Development of both macrophages and monocytes is dependent on receptor signalling mediated by the colony stimulating factor 1 receptor (CSF-1R) also known as M-CSF receptor (**Fig. 2**) (52). Deficiency in M-CSF receptor results in a host that is unable to develop a monocyte population and develops monocytopenia (126-128). These mice are unable to respond to two key growth factors for monocyte and macrophage development, M-CSF and IL-34, both of which bind to M-CSF receptor (**Fig. 2**) (90). In the bone marrow, common myeloid progenitors (CMPs) and macrophage-DC progenitors (MDPs) maintain their ability to differentiate into either monocytes/macrophages or DCs

but the latter lose their ability to differentiate into neutrophils (**Fig. 4**) (129, 130). Circulating monocytes that are derived from MDPs can differentiate into either MoDCs or monocyte derived macrophages, which will depend on the stimulatory conditions present in the peripheral tissue (i.e. inflammation) (**Fig. 4**) (70, 131). Common DC progenitors (CDPs) maintain their potential to differentiate into conventional DCs and pDCs while maintaining high expression of M-CSF and FLT3L with low stem cell factor receptor c-Kit (CD117) (**Fig. 4**) (132, 133). Pre-DC progenitors differentiate from CDPs and lose their ability to differentiate into pDCs but maintain their ability to become conventional DCs (**Fig. 4**) (134, 135). Pre-DCs seed into lymphoid and non-lymphoid organs and differentiate into CD8 α and CD11b DCs (**Fig. 4**). Indeed, with such distinct lineages that distinguishes monocytes and macrophages from different DC subsets, the use of ontogeny to classify different mononuclear phagocytes has recently been proposed (50).

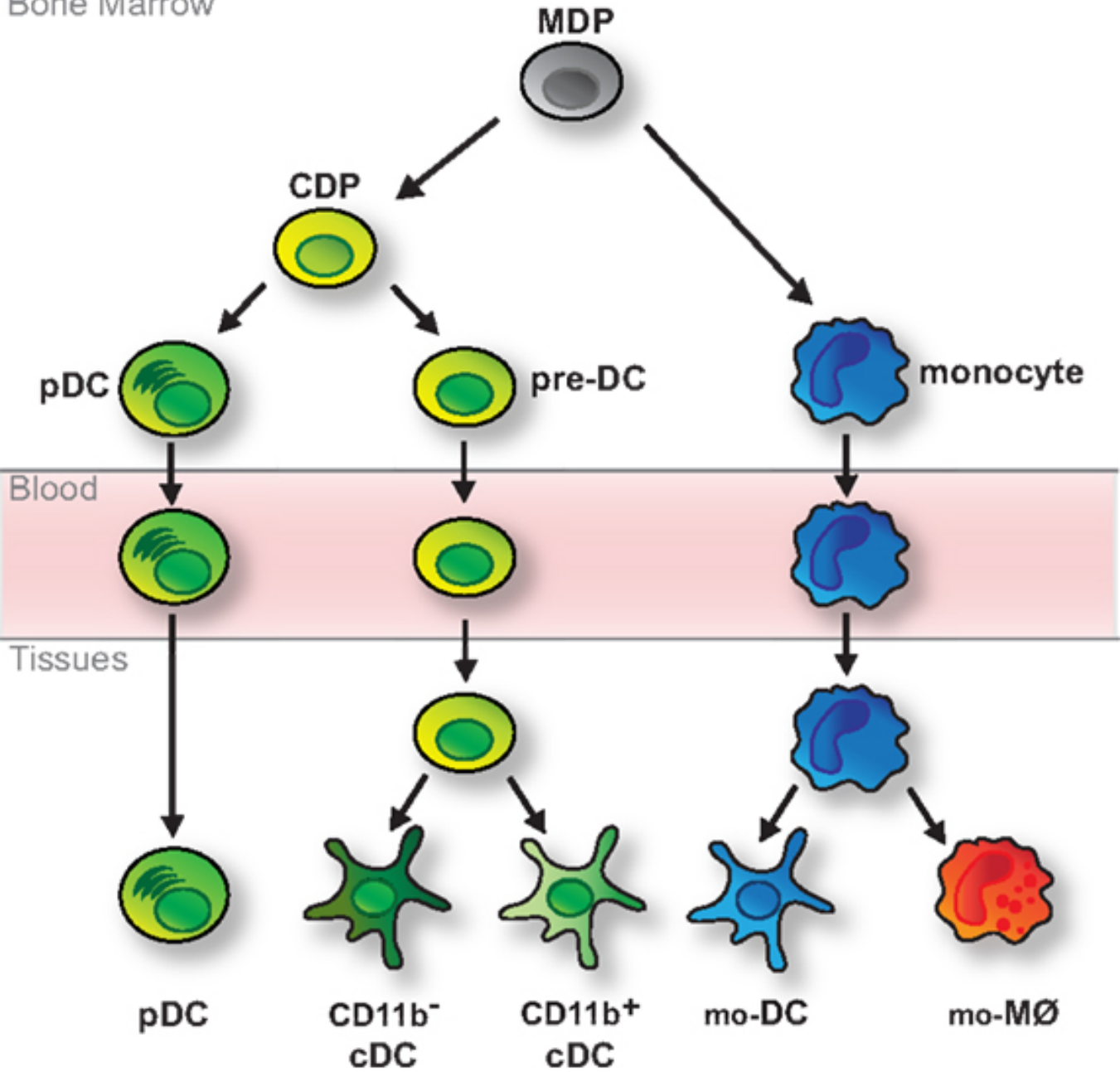
Tissue resident macrophages have a longer half-life (20 days) in comparison to monocytes or DCs (3-5 days) (76, 136). This is in accordance with the fact that macrophages acquire tissue resident functions (137). In contrast, DCs are short lived and maintain a quiescent state (137). For instance, CD8 α DCs and CD103⁺ DCs represent two distinct populations of DCs that reside in lymphoid organs or peripheral tissues respectively (52, 101, 106, 125). Despite residing in distinct tissues, both populations maintain similar transcriptional signatures and as a consequence have similar functional properties (137, 138). DCs and macrophages require FLT3L and M-CSF respectively for proper

Figure 4: Mononuclear phagocyte development

DCs and monocytes/macrophages can arise from macrophage-DC precursor (MDPs) differentiated from hematopoietic stem cells (HSCs). MDP can further differentiate into monocytes (that can yield macrophages and monocyte derived DCs under various inflammatory conditions in the peripheral tissues) and CDPs to generate various DC subsets. CDPs can differentiate into two distinct lineages, which include pDC and pre-DCs. The latter will circulate from the bone marrow to peripheral tissues and differentiate into CD8 α and CD11b DCs.

Figure as originally published in Poltorak MP and Schraml BU (2015) Fate mapping of dendritic cells. Front Immunol 6:199 doi: 10.3389/fimmu.2015.00199.

Bone Marrow



development from progenitor cells (52). Development of DC subsets from bone marrow progenitors is highly dependent on FLT3 receptor signalling and is independent of M-CSF receptor signalling (78, 79, 139). Furthermore, administration of FLT3L dramatically increases the number of DCs *in-vivo* (77, 140). Other growth factors such as GM-CSF contribute to DC development and acquisition of DC function (141), however, FLT3 receptor signalling is the main determinant for DC development (139).

The human subset of DCs has been recently characterized and murine counterparts have been discovered based on immune function, shared surface markers and transcriptional factors critical for development (142). Unfortunately, human DC subsets do not share many common markers with their murine counterparts (142, 143). However, DC subsets sharing common functional roles in the innate and adaptive immune response in humans have been uncovered (142, 143). Human DCs isolated from the blood are generally characterized as BDCA1⁺ DCs (also express CD1c), BDCA3⁺ DCs (also express CD141) and pDCs (144-146). BDCA3⁺ DCs express high levels of XCR1 and CLEC9A and maintain the ability to cross-present exogenous antigens such as cellular debris from dead cells (147-149). As a result, BDCA1⁺ DCs are considered homologous to murine CD8 α and CD103⁺ DCs (148, 150). Additionally, human BDCA1⁺ DCs closely resemble murine CD11b DCs (142). Human skin resident DCs have also been characterized as Langerhans cells, CD1a⁺ dermal DCs and CD14⁺ dermal DCs (142). In stark contrast to their murine counterparts, both human Langerhans cells and CD1a⁺ dermal DCs have the capacity for cross-

presentation (151, 152). Deciphering the roles of human subsets can provide insights into novel therapeutics and vaccine strategies for highly relevant pathogens and cancers.

1.10 *Salmonella* Pathogenesis

Phagosomal pathogens such as *Salmonella*, *Mycobacteria* and *Leishmania* engage an array of virulence factors to evade the immune response, resulting in a chronic infection (153). *Salmonella* promotes a wide range of diseases from enterocolitis and typhoid to even inflammatory bowel disease and cancers (154). Non-typhoidal *Salmonella* causes self-limiting colitis in most individuals and a fatal disease in immunocompromised individuals, such as HIV patients in sub-Saharan Africa. (155). Many typhoid strains of *Salmonella* are acquiring drug resistance, and there is no effective vaccine against many clinically relevant typhoidal and non typhoidal *Salmonella* strains (155), emphasizing the need for novel therapeutics and better understanding of the pathogen.

Infection of susceptible mouse strains with *Salmonella enterica subsp. enterica serovar* Typhimurium (ST) causes a systemic disease that closely resembles typhoid fever in humans (156). Early immune control of ST infection is mediated by the innate immune system, particularly by mononuclear phagocytes (155). Innate control against ST depends on the engagement of PRRs such as TLRs (157), in addition to an appropriate pro-inflammatory response mediated by IFN- γ (158) and TNF- α (159). Susceptible strains of mice, such as C57Bl/6 (B6)

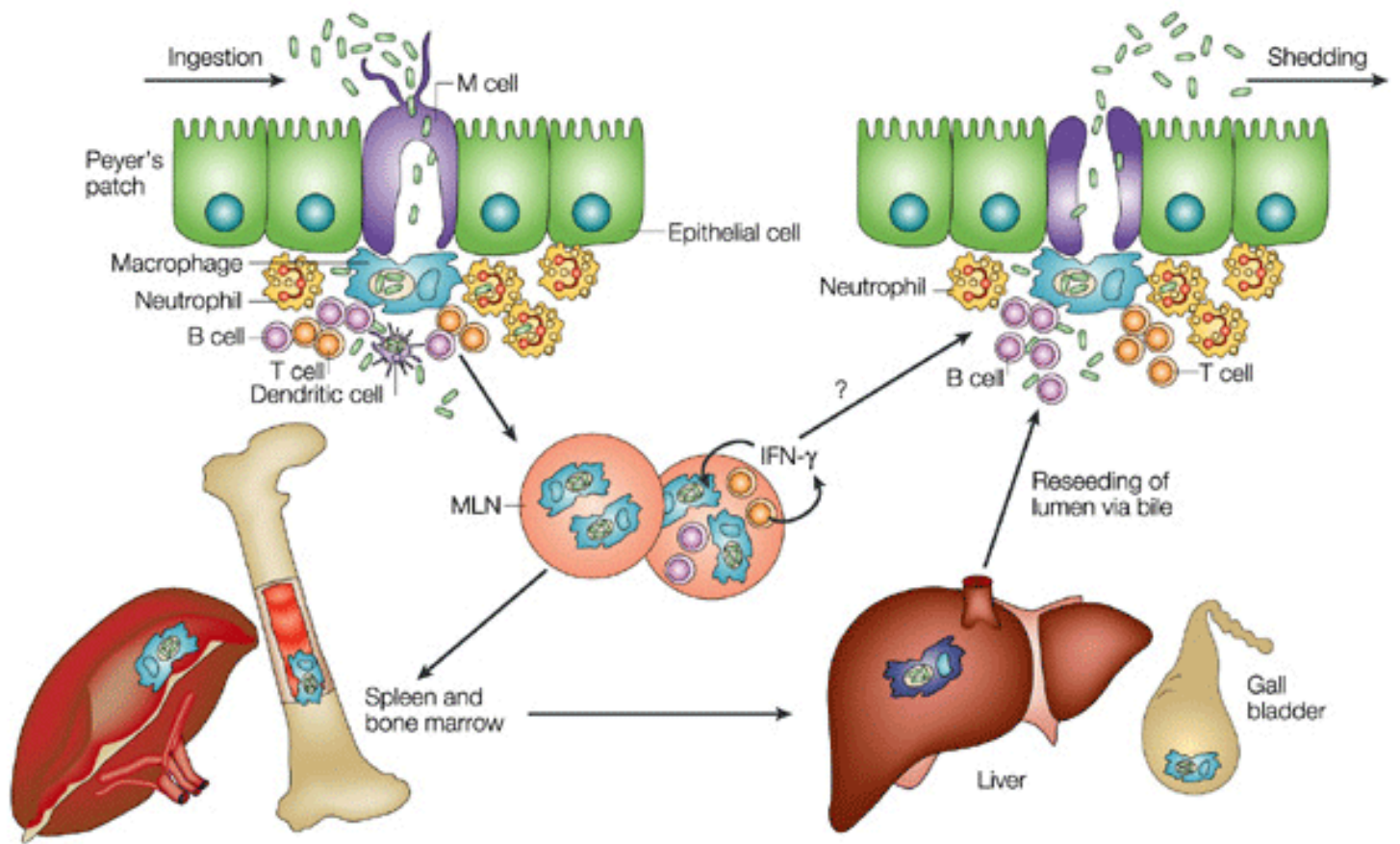
mice, succumb to infection within seven days while mouse strains such as the 129SvJ maintain a chronic state of infection and the pathogen is never effectively eliminated (160, 161). Thus, the mouse model of ST is ideal for dissecting the mechanisms of immune failure against phagosomal pathogens.

Following infection through the oral route, ST can penetrate the mucosal barrier of the gastrointestinal tract by actively infecting Microfold cells (M cells), which are specialized immune cells located in the mucosa-associated lymphoid tissue (MALT) such as in the Peyer's patches (**Fig. 5**) (154). ST thrives in phagocytic cells such as epithelial cells, macrophages and DCs (154, 162). Macrophages and DCs are critical in controlling the bacterial burden of ST during the early phase of infection (**Fig. 5**). Following infection, macrophages and DCs are rapidly eliminated by the bacterium to promote immune evasion and pathogenesis (**Fig. 5**) (163, 164). ST utilizes a type-three secretion system (T3SS) apparatus to deliver virulence factors into the cytoplasm of host cells (165). Virulence factors located in the Salmonella pathogenicity island (SPI)-1 induce uptake in epithelial cells, while relying on active phagocytosis for internalization by macrophages and DCs (166). Other virulence factors of Salmonella that are located in the SPI-2, and are also delivered through the T3SS, promote intracellular survival by creating a hospitable niche in the phagosome, allowing for bacterial survival and proliferation in an environment usually reserved for pathogen elimination (167, 168). Experiments utilizing various mutants of ST have demonstrated that intracellular replication within infected cells is essential for its virulence (169).

Figure 5: *Salmonella* Typhimurium

Salmonella enterica serovar Typhimurium (ST) is a facultative intracellular bacterium that causes gastroenteritis in humans by infection through the oral route in cases of ingestion of contaminated food. ST can infect Microfold cells (M cells), which are special cells located in the Peyer's patches of the intestinal tract that facilitate transfer of luminal antigens to phagocytic immune cells through a process called transcytosis. Specialized immune cells such as neutrophils, macrophages and DCs as well as adaptive immune cells are recruited to this microenvironment to eliminate the invading bacterium. In certain cases, ST can utilize immune cells such as DCs to spread systemically to the draining mesenteric lymph nodes (MLNs) and peripheral tissues (such as the gall bladder, liver, spleen, bone marrow) in the host leading to systemic salmonellosis (such as typhoid fever). Pro-inflammatory cytokines such as IFN- γ and TNF- α are critical for host protection against ST.

Figure as originally published in Monack DM et al. (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol 2:9.



1.11 Innate Immune Response against *Salmonella*

TLR recognition of ST is critical for innate immune responses against the bacterium. Four TLRs recognize PAMPs associated with ST: TLR2 (lipoprotein), TLR4 (LPS), TLR5 (flagellin) and TLR9 (CpG ODN) (155). Amongst these TLRs, TLR4 signalling is critical for primary ST infection (157, 170). The impact of TLR2, TLR5 and TLR9 signalling on promoting ST immunity is apparent only when TLR4 is disabled (171). ST can also utilize TLR9 signalling in the phagosome to enable virulence factors and induce pathogenicity (171). Disabling all TLR signalling pathways (while maintaining MyD88 signalling for IL-1 receptors) leads to defective intracellular replication of ST within macrophages, and enhanced extracellular replication subsequent to lack of TLR mediated recruitment of innate immune cells (172). Thus, TLR signalling mediates immune protection against ST. However, certain TLR signalling pathways are utilized by ST to promote the induction of virulence factors and bacterial dissemination (171-173).

Engagement of caspase mediated inflammation is an important mechanism of innate immune defence against ST (174). Activation of the multimeric protein complex, known as the inflammasome, is critical for activation and release of IL-1 β , IL-18 through the induction of inflammatory cell death known as pyroptosis (174). Indeed, mice deficient in IL-18 show slightly enhanced susceptibility to ST infection (175). ST is also detected by multiple NLR receptors by binding to common ST motifs localized in the cytosol. For instance, neuronal apoptosis inhibitor protein (NAIP)5 and NAIP6, in combination with

NLRC4, detect cytosolic flagellin and T3SS rod protein PrgJ expressed by ST leading to activation of the inflammasome (13, 176, 177). Although the specific ligand is still unclear, ST can also engage NLRP3 activation leading to inflammasome activation (155). Some redundancy in NLR signalling against ST is apparent as enhanced susceptibility is only observed in mice deficient in both NLRC4 and NLRP3, in comparison to a modest susceptibility in single knockout mice (176). Therefore, NLR activation is critical for immune protection against ST by mediating inflammasome activation and the release of DAMPs and processed inflammatory cytokines.

Natural resistance associated macrophage protein 1 (*Slc11a1*) is a key resistance gene that induces innate immune protection against various intracellular bacteria that reside in the phagosome as a mechanism of immune evasion, such as Mycobacteria and Salmonella (155, 178). Nramp1 is a protein expressed in macrophages and DCs associated with removal of ions from the phagosome (179). The difference in pathogenesis between B6 and 129SvJ mice strains is considered to be attributed to a mutation in the *Nramp1* allele in the B6 strain (180). Nramp1 encodes a lysosomal transmembrane protein which transports divalent cations across the phagosomal membrane in macrophages (181). Furthermore, Nramp1 expression induces the expression of the peptide lipocalin-2 (LCN2), a scavenger of bacterial siderophore that limits bacterial proliferation within the cell (182). Susceptible B6 mice have been shown to express a mutation in the *Nramp1* gene (*Nramp1^{169D}*) which results in rapid host fatality (178-180). In contrast, resistant strains of mice express a functional copy

of *Nramp1* (*Nramp*^{169G}), which allows them to survive infection during the initial stage (179, 183). However, other genes associated with host survival in resistant mice have been described (184, 185). B6 mice expressing a functional *Nramp1* allele survive for a longer period of time, but still succumb to ST infection (171). *Nramp1* polymorphisms are also associated with disease susceptibility in the human population (155).

1.12 Interferons

In 1957, Isaacs and Lindenmann used heat-killed influenza to induce soluble factors that were shown to inhibit infection with live influenza virus, which were known as Interferon (IFN) (186). Overall, there are three types of IFNs: IFN-I, IFN-II and IFN-III that are distinct in function, receptor recognition and intracellular signalling. There are 13 types of IFN-I in humans and 14 in mice, which include one IFN- β and numerous IFN α , all of which bind with different affinities to the receptor IFNAR (187). IFN-I promotes cells to engage an antiviral state by translating proteins that promote RNA degradation and induce cellular apoptosis in the infected cell (188). IFN-II consists of one cytokine: IFN γ while IFN-III includes IFN λ 1, IFN λ 2 and IFN λ 3. Both IFN-I and IFN-II have receptors expressed on a broad range of cells, while IFN-III is restricted to a few types of cells such as epithelial cells (189).

IFN-I include both IFN α and IFN β , which generally act as anti-viral factors (190). All virally infected cells produce IFN β . In contrast, IFN α is produced by pDCs and APCs such as macrophages and DCs. pDCs make the most IFN α on

a per cell basis (191). However, following depletion of pDCs, there is still production of IFN α which indicates that other cell types are able to compensate (192). Both IFN α and IFN β bind to a heterodimer receptor IFNAR, composed of IFN α R1 and IFN α R2 that is ubiquitously expressed on most cells (190). IFNAR signalling is mediated by Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which is propagated through transcription factors signal transducer and activator of transcription (STATs) and interferon-regulatory factor (IRF)9 following viral or bacterial stimulation (through TLRs and NLRs) (190).

Apart from mediating anti-viral immunity, IFN-I can also impact APC development, maturation and antigen presentation (193). Measles virus and LCMV evade immune surveillance by inhibiting DC development through interference with IFN-I mediated STAT2 signalling (194). In addition, transcription factors IRF4 and IRF8 have been shown to be critical for CD11b and CD8 α DC development independent of IFN-I signalling (195). Furthermore, IFN-I has been shown to enhance immune function by acting synergistically with growth factors such as GM-CSF on DC (196, 197).

IFN-I signalling can also influence DC maturation and function and can have major implications on T cell responses during infections (193). IFN-I has been shown to up-regulate activation markers and co-stimulatory molecules such as MHC-II, CD40, CD80, CD86, CCR5 and CCR7 (198, 199). Furthermore, IFN-I can act as a key activation signal (cytokine mediated signal 3) to promote T cell priming (200, 201). IFN-I also induces migration of primed cells to the draining lymph nodes through LFA-1 (199) and chemokine production such as CXCL9

and CXCL10 (202). IFN-I engages various STAT signalling homodimers and heterodimers which can result in different outcomes (203, 204). For instance, STAT1 promotes pro-inflammatory, anti-proliferative and pro-apoptotic pathways limiting viral spread. In contrast, STAT3/4/5 heterodimers promote cell survival, proliferation and differentiation (205, 206).

IFN-I has been shown to enhance antigen processing and presentation (111, 207, 208) and increase priming of T cells in secondary lymphoid organs (209). Furthermore, when chronic pathogens induce prolonged exposure to IFN-I, DCs can acquire immunoregulatory functions through the expression of PD-L1 and IL-10 to mediate immune suppression (210). In certain cases, immune cells respond to pathogens by shutting down IFN-I signalling to promote immune responses. For instance, ubiquitin-specific peptidases (USP)18 metallophilic macrophages (MMPs) promote acquisition of pathogenic antigen by turning off IFN-I signalling to allow viral replication, which can be transferred to other APCs to promote cross-presentation and CD8⁺ T cell priming (211). Furthermore, IFN-I enhanced antigen presentation in the specialized subset of CD8 α DCs, indicating some impact on cross-presentation (28, 212). For instance, administration of IFN- α with OVA protein boosts cross priming of OVA specific CD8⁺ T cells (208, 213). This has been shown to be mediated through delayed acidification of phagosomal content and routing proteolytic contents towards MHC class-I loading to promote cross-presentation of antigens (111, 214, 215).

1.13 Programmed Cell Death

Programmed cell death is essential for many physiological processes, such as development, cellular renewal, regulation of immune populations as well as an important response to stress, tissue damage and infection (216). Engagement of an appropriate cell death pathway can mediate immune protection by limiting pathogenic dissemination, secretion of inflammatory cytokines as well as release of “alarmins” to alert neighbouring cells of a pending infection (216). However, many pathogens have evolved strategies to manipulate various cell death pathways to promote pathogenicity and dampen the immune response (217).

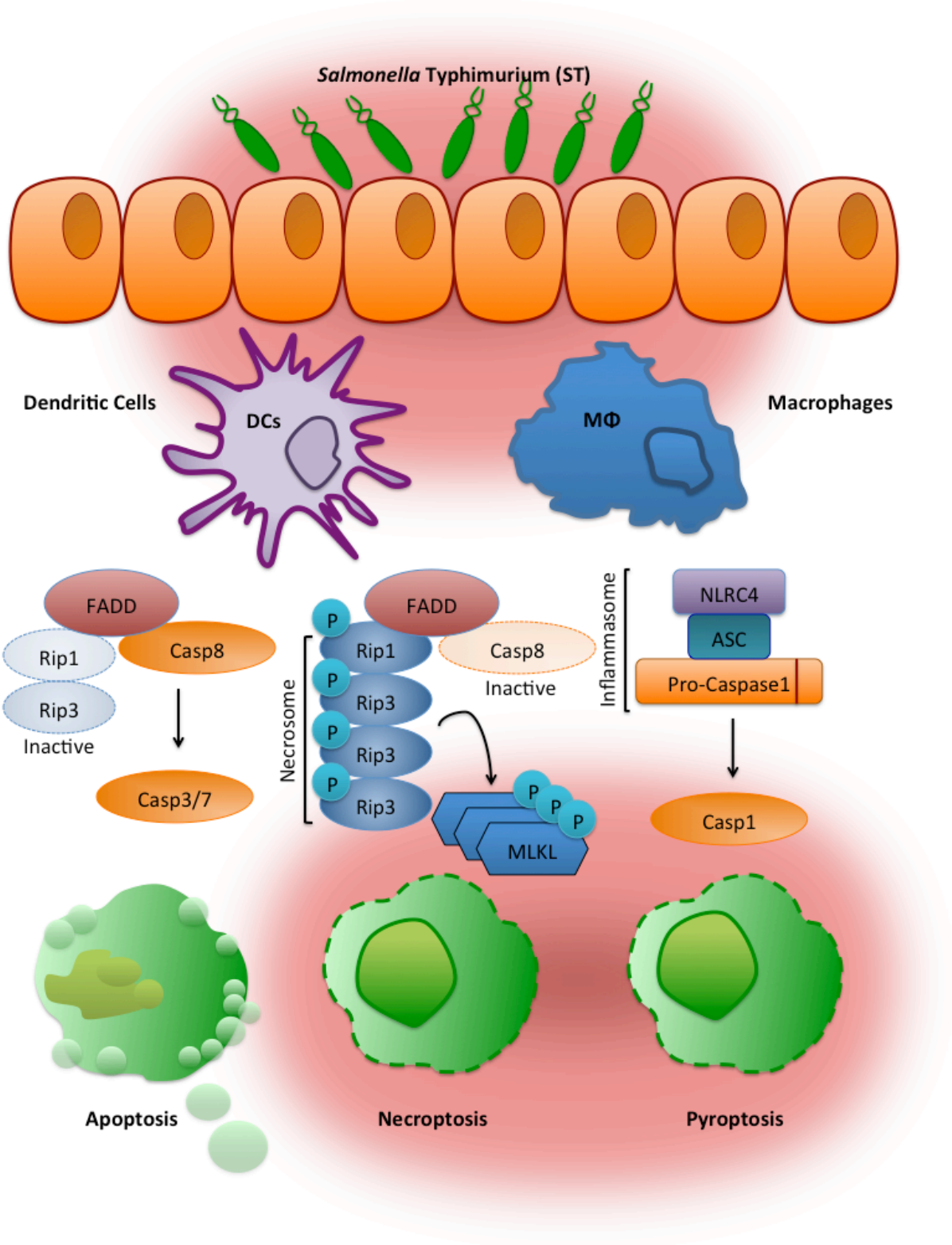
Cell death is classically known to follow two distinct pathways: anti-inflammatory cell death through apoptosis and inflammatory cell death through necrosis (217). Programmed cell death was thought to be non-inflammatory and occur through the apoptosis pathway, characterized by membrane blebbing and fragmentation (218, 219). Apoptosis has been shown to be critical during development and embryogenesis. Furthermore, apoptotic blebs and cellular debris are rapidly cleared by macrophages through a process known as “efferocytosis” (94, 113, 220, 221). In addition, DCs have been shown to also acquire antigenic blebs from cells that have engaged apoptosis to promote antigen presentation and T cell priming (113). Importantly, apoptosis does not appear to promote inflammation as cellular contents are contained within cellular membranes (113). Cell death, tissue damage and inflammation are often pervasive during virulent infections (222). In circumstances where cell death occurs accidentally, such as during stress or mechanical injury, cells die through

necrosis (223). Engagement of necrosis results in oncosis (cell death with swelling) and release of cytoplasmic contents into the extracellular milieu, resulting in the release of cellular contents, including DAMPs such as HMGB-1, IL-1 α and ATP which is considered to be highly inflammatory (223).

Recently discovered alternative forms of cell death such as necroptosis and pyroptosis promote inflammation by facilitating the processing of various inflammatory cytokines and release of numerous intracellular DAMPs (**Fig. 6**) (217, 224, 225). Necroptosis is induced by phosphorylation of the receptor interacting protein kinase (RipK)1 following TNF-R, Interferon alpha receptor (IFNAR) or IFN γ R engagement (226-231). Phosphorylated RipK1 leads to an interaction with RipK3 and the formation of the necrosome (232), which can result in phosphorylation of the pore-forming mixed lineage kinase domain-like (MLKL) trimer (233). The MLKL trimer then translocate to the plasma membrane to facilitate membrane rupture, cell lysis and the release of intracellular DAMPs leading to inflammation through necroptosis (**Fig. 6**) (234, 235). In addition to mediating necrosome formation, RipK3 can also facilitate processing of inflammatory cytokines such as IL-1 β , providing a critical link between cell death and the induction of inflammation (236, 237). Pyroptosis is a cell death pathway engaged following the assembly of the inflammasome (**Fig. 6**) (238). The inflammasome consists of an NLR sensor, an oligomerization domain and an effector domain, which induces recruitment, autocleavage and subsequent activation of Caspase-1, which in turn cleaves inflammatory IL-1 cytokines such as pro-IL-1 β and pro-IL-18 into their active forms (239). Pyroptosis also results in

Figure 6: Pathways of cell death

Salmonella Typhimurium (ST) is a virulent intracellular pathogen that is rapidly engulfed by macrophages and dendritic cells upon breaching the epithelial layer in the gut. Cell death by infected cells is a critical mechanism of pathogen control. The cell death pathway engaged by infected cells is critical for proper immune protection. Programmed cell death can occur through apoptosis (Casp3/7 mediated cell death), necroptosis (RipK1/RipK3 mediated programmed necrosis) or pyroptosis (Caspase1 mediated inflammatory cell death) mediated by NOD-like receptors such as NLRC4.



host cell lysis, facilitating the release of IL-1 α and processed IL-1 β and IL-18 (238). Unlike most conventional cytokines and chemokines, IL-1 cytokines lack a signalling sequence and thus rely on cell death pathways such as pyroptosis that releases cytoplasmic content for secretion (240). However, other mechanisms of IL-1 β secretion have been discovered but play a minor role in comparison to release following pyroptosis (241).

1.14 Apoptosis

The term Apoptosis was first coined by John Kerr in 1972 as a “mechanism of controlled cell deletion” (218). Apoptosis comes from the Greek word meaning “falling off”, pertaining to the bleb formation following engagement of apoptotic cell death. Apoptosis is a regulated form of cell death that is critical for proper development, homeostasis and immune regulation. By maintaining intracellular content within membrane blebs, apoptosis is considered to be non-inflammatory (113). In fact, these apoptotic blebs have been shown to provide antigen to DCs to initiate an immune response (113).

The molecular and genetic mechanism of apoptosis was first deciphered using the hermaphrodite worm model *Caenorhabditis elegans*. During the developmental stages leading to an adult worm, cellular divisions should yield a total of 1,090 somatic cells (242). However, the adult worm consists of 959 cells, indicating that 131 cells are deleted through programmed cell death (242). Genetic screens uncovered four proteins that are critical for programmed cell death, and proper development of the worm: Egl-1, Ced-4 and Ced-3 (243). The

mammalian homologues of these proteins are the pro-apoptotic BH3, the adapter apoptotic protease activating factor-1 (APAF)-1 and cysteine proteases known in other species as caspases, which are critical components to promote apoptosis in mammalian cells (243).

Apoptosis is mediated by caspases, which exist as single chain inactive zymogens that are processed either through proximal activation or cleavage into their active forms (219, 244). Apoptotic caspases can be characterized into two groups: initiator caspases (Caspase-8, -9) that propagate intrinsic and extrinsic cell death signals and executioner caspases (Caspase-3, -6, -7) that mediate terminal processes of cell death (219, 245). Additionally, inflammatory caspases such as Caspase-1, -4, -5 and -11 are critical for inflammasome activation but do not play any characterized role in cell death through apoptotic mechanism (246, 247).

Apoptosis can be initiated through the intrinsic and extrinsic apoptotic pathways (219). The extrinsic pathway can be triggered by transmembrane death receptors such as FAS (CD95), TNFR1 and TNF-related apoptosis inducing ligand (TRAIL), which results in the activation of the initiator Caspase-8 in a complex known as the death-inducing signaling complex (DISC) (216, 248). Caspase-8 then in turn cleaves the executioner Caspase-3, which results in apoptotic cell death (219, 249). Alternatively, apoptosis can be initiated by an intrinsic mechanism in which case DNA damage, infection or developmental signals results in the permeabilization of the mitochondrial membrane that results in the release of cytochrome c (250-252). This results in the formation of the

muliprotein complex known as the apoptosome, consisting of cytochrome c, APAF-1 and pro-Caspase-9 (253). Both Caspase-8 (extrinsic pathway) and Caspase-9 (intrinsic pathway) leads to cleavage and activation of Caspase-3, -6 and -7 leading to apoptotic cell death (219, 245).

1.15 Necroptosis

Necroptosis, or programmed necrosis, was first described as a cellular response to TNF signalling (217, 254). Commonly, TNF signalling leads to nuclear factor- κ B (NF- κ B) activation and production of pro-inflammatory cytokines (230). However, in cases where cellular inhibitors of apoptosis proteins (cIAPs)-1/2 or Caspase-8 protein levels are limiting or their function is inhibited, the necroptosis death pathway is engaged (229, 254-258). Viral inhibitors of Caspase-8 such as cytokine response modifier A (CrmA) and baculovirus p35 can limit induction of apoptosis but as a consequence induces necroptosis to mediate an alternative mechanism of pathogen control (259). Necroptosis can be induced by a variety of stimuli, such as cellular stress or infection (229, 230). Necroptosis has been shown to be critical in mediating immune protection against various pathogens. For instance, Mouse Cytomegalovirus (MCMV) encodes a viral protein M45 that has a receptor-interacting protein homotypic interaction motif (RHIM) domain that binds to RipK1 and RipK3, essentially inhibiting necroptosis and mediating immune evasion (260-262). Furthermore, Human Cytomegalovirus infection is shown to inhibit necroptosis through an unknown pathway by reducing RipK3 and MLKL activation (263).

Activation of TNFR1 leads to recruitment of a multi-protein survival complex known as complex-I, which includes TNF receptor associated factor (TRAF)-2 and TRAF-5, cIAP1/2 and RIPK (264). This complex is regulated by the ubiquitination of RipK1, TRAF-2 and TRAF-5 by cIAP1/2, leading to activation of NF- κ B signalling and subsequent pro-survival state (264). Alternatively, deubiquitination of RipK1 by A20 or cylindromatosis (CYLD) can lead to complex II formation, which consist of TNF receptor type 1 associated death domain (TRADD), Fas-associated protein with death domain (FADD), RipK1 and RipK3 and capsase-8 (264). This complex leads to Caspase-8 activation and cleavage of RipK1 and RipK3 resulting in the induction of apoptosis (264, 265). The presence of cellular FADD-like IL-1 β -converting enzyme (cFLIP) in this complex leads to inhibition of Caspase-8 processing, cleavage of RipK1 and RipK3 and promotion of NF- κ B and survival (266, 267). In the absence of Caspase-8 or cIAP1/2, both RipK1 and RipK3 are phosphorylated leading to the formation of the necrosome complex that leads to the induction of necroptotic cell death (268).

Necroptosis is described as a pathway that is engaged following the formation of the necrosome, which consist of a dimerization complex between RipK1 and RipK3 (229). Under steady state conditions, Caspase-8 normally cleaves RipK1 and RipK3 to limit necroptosis (269). In conditions where Caspase-8 is limiting or inhibited, RipK1 can be auto-phosphorylated on the serine 161 residue, leading to phosphorylation of RipK3 and the formation of the necrosome (269, 270). Following RipK1-RipK3 dimerization, RipK3 can induce auto-phosphorylation and the formation of an amyloid structure (232). However,

the exact mechanism of necrosome formation and subsequent signalling that leads to necroptotic cell death is still under investigation (271). Necroptosis can be blocked by the RipK1 kinase inhibitor Necrostatin-1 (Nec-1), underlining the importance of RipK1 in initiating the necrosome complex (272). However, necroptosis has been shown to occur through RipK1 dependent and independent pathways (271, 273). Necrosome formation leads to the phosphorylation of the pseudokinase MLKL, leading to a conformational change that exposes the four helical bundle domains and subsequent cell lysis (274, 275). This can occur either through MLKL acting as a platform for recruiting ion channels inducing an influx of ions (233) or through MLKL directing pore complexes in the plasma membrane (276, 277), leading to the release of DAMPs and pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-18 or HMGB1) (274, 278).

1.16 Regulation of Apoptosis and Necroptosis

Caspase-8 is an initiator caspase that, along with FADD and cFLIP participates in forming the multi-protein complex known as the DISC following engagement of the TNFR family of receptors (265). Caspase-8 has two death effector domains (DED), which can facilitate homotypic dimerization and subsequent proximal autocleavage (241, 279). Caspase-8, like all other caspases, exists as a zymogen. Activation of Caspase-8 requires cleavage at specific residues (D374, D384, D216) allowing the protein to stabilize following the removal of the amino acids that consist of the pro-domain, into the large (p18) and small (p10) subunit (241). Activated Caspase-8 can now proceed to cleave effector caspases such as Caspase-3 to induce apoptotic death (280, 281).

In addition to its critical role in dictating cell death pathways following TNF signalling, Caspase-8 has also been shown to play a role the activation of inflammatory cytokines by mediating pro-IL-1 β processing (241). Proteases other than Caspase-1 are capable of cleaving IL-1 β , such as elastase, collagenase, and cathepsins (282-284). Caspase-8 has been shown to cleave pro-IL-1 β into its active form independent of Caspase-1 upon stimulation of TLR3 and TLR4 following upregulation of TRIF (285). Overexpression of Caspase-8 leads to enhanced cleavage and processing of pro-IL-1 β , at the same cleavage site as Caspase-1 (282, 285). Furthermore, pro-IL-1 β processing following treatment with chemotherapeutic agents doxorubicin and staurosporine leads to robust IL-1 β release independent of Caspase-1, NLRP3 and NLRC4 (286).

Caspase-8-deficient mice do not survive past the embryonic stage E10.5 due to vascular abnormalities induced by unregulated necroptosis (287). This phenotype is similar in FADD-deficient and cFlip-deficient mice (288, 289). cFlip inhibits Caspase-8 autocleavage and in the process promotes cleavage of both RipK1 (preventing the formation of the necrosome) and CYLD (266, 290). Thus loss of Caspase-8 activity, through viral inhibitors or genetic deletion promotes necroptotic cell death (259, 264, 287). However, mice deficient in both Caspase-8 and RipK3 in concert are viable due to the ablation of both apoptosis (Caspase-8) and necroptosis (RipK3) pathways (269). As a result these mice eventually develop a lymphoproliferative disease due to the inability to delete immune cells (258).

Cellular inhibitors of apoptosis (cIAP)-1 (*Birc2*) and cIAP-2 (*Birc3*) and the X chromosome-linked IAP (XIAP, *Birc4*) are a family of proteins critical to regulating apoptotic cell death (291, 292). XIAPs can directly bind to Caspase-3, -7 and -9 to inhibit induction of apoptotic cell death (293, 294). In contrast, cIAPs inhibit apoptotic caspases by binding to specific conserved baculoviral IAP repeat motif and promote ubiquitination of the caspase substrate (295, 296). By utilizing their ubiquitin ligase properties, cIAP1/2 can promote ubiquitination through their RING domain finger E3 ligase domain and subsequent degradation through K48-polyubiquitination linkages (297-299). In contrast, ubiquitination of targets through K63-polyubiquitination linkages such as RipK1, TRAF2 and TRAF5 mediated by cIAP1/2 can also promote cell survival and NF- κ B signalling pathways (300-302). Interestingly, a class of pharmacological agents known as second mitochondria-derived activator of caspases (SMAC)-mimetics are able to inhibit XIAP function and induce degradation of cIAP1 and cIAP2 (303). Genetic deficiency or pharmacological inhibition of cIAP1/2 leads to loss of cell survival and engagement of apoptosis (298, 299, 304). Many types of cancers rely on overexpression of cIAPs to limit induction of cell death and thus SMAC mimetics have been explored as a therapeutic avenue to treat cancer patients, which is currently in clinical trials (296).

Interactions between different adapter proteins, whether it is for a transient interaction or for assembly of a functional complex, can occur through distinct domains expressed by each protein within a particular complex. These domains include: pyrin domains, CARD domain, death domains (DD), and DED (305-307).

RIP kinases are serine/threonine kinases that are critical for regulation of cell death (through necroptosis or apoptosis) and survival (NF- κ B signalling) (230, 308). The formation of the necrosome, the dimerization complex between RipK1 and RipK3, is critical for engagement of necroptotic cell death (236). RipK1 contains a death domain, which allows it to recruit large complex of proteins for signalling (309). For instance, during genotoxic stress and loss of XIAP, cIAP1 and cIAP2; RipK1 interacts with FADD and Caspase-8 to form the ripoptosome, which promotes apoptosis independent of TNFR signalling (266, 267). Both RipK1 and RipK3 contain a RIP homotypic interaction motif (RHIM) domain, which allows both molecules to interact with each other to form the necrosome and mediate necroptosis (309). Nec-1 was an important tool that revealed the critical role of RipK1 kinases activity and its role in promoting necroptosis (272, 310). RipK1-deficient mice are perinatal lethal and succumb to excessive inflammation (311). Interestingly, RipK1 deficiency in just the kinase region of the protein (RipK1-kinase dead) leads to normal development of the mouse, underlining the important structural roles, such as scaffolding properties of RipK1 to promote cell survival (312-315).

RipK3 is a critical component of the necrosome that, along with RipK1 mediates necroptosis (309). RipK3-deficient mice are viable and show normal NF- κ B signalling and are unable to induce necroptosis (316). Interestingly, RipK3 kinase deficient (RipK3D161N-kinase dead) mice are embryonic lethal (due to excessive RipK1-FADD-Casp8 apoptosis) (315), but RipK3K51A are viable (317), emphasizing the complex regulation of cell death mediated by RipK3

kinase and scaffolding properties. The catalytic properties of RipK3 promotes necroptosis, while the scaffolding properties of RipK3 can promote apoptosis (315). Furthermore, RipK3 has been shown to also play a critical role in the activation of inflammatory cytokines such as IL-1 β by promoting the Caspase-1 inflammasome independent of necroptosis (237, 241, 318).

1.17 Pyroptosis and Inflammation

Pyroptosis is an inflammatory form of cell death that is triggered by pathogenic bacteria such as Salmonella (163, 225). Cell death through pyroptosis requires activation of Caspase-1 or Caspase-11 (to homologues Caspase-4 and -5 in humans) (319, 320). Caspase-1 mediated pyroptosis requires the assembly of the inflammasome (225). Formation of the inflammasome can be mediated by a variety of putative NLR sensors such as NLRP3, NLRC4, AIM2 and NLRP1 (238). NLR activation leads to ASC self-association and the formation of a large complex of 1 μ m commonly referred to as a speck, which recruits pro-Caspase-1 (321). The inflammaosome is a group of multimeric protein complex consisting of an inflammasome sensor (NLRC4, NLRP3), an adapter protein (ASC) and Caspase1 (238). In addition, inflammasome activation can lead to pyroptosis (238). Interestingly, post translational modifications of NLRs, such as phosphorylation and ubiquitination have also been shown to regulate inflammasome activation (322-324).

The ASC contains two death fold domains, one containing a pyrin domain and another consisting of a CARD domain (325). ASC interacts with the

inflammasome sensor using the pyrin domain (325). This leads to the assembly of ASC into a large protein spec of multimers of ASC dimers (326, 327). Through the CARD domain, ASC assembles with capase-1, bringing them in close proximity to induce capsase1 self cleavage and activation of Caspase-1, which then leads to activation of proteins such as pro-IL-1 β and pro-IL1 α (326, 327). Interestingly, Caspase-8 can also be associated with the inflammasome either with or without Caspase-1 (328).

Historically, inflammation was characterized by the mechanisms that induce redness, heat, swelling and pain (329). The innate and adaptive immune system is capable of mediating inflammation by secreting pro-inflammatory cytokines and chemokines, which results in permeabilization of blood vessels and recruitment of other immune cells to the site of infection or injury (10). This response can leads to proper control of pathogens or in some cases, tissue injury (10). In certain cases, the inflammatory response is exacerbated, leading to immune mediated inflammatory diseases such as arthritis (330, 331). Inflammation is mediated by various PRRs expressed by immune cells such as TLRs and NLRs (10). These receptors recognize pathogenic (i.e. LPS, flagellin, viral DNA) or non-pathogenic (i.e. ATP, nigericin) ligands, leading to inflammatory responses against pathogens, sterile inflammation or tissue injury (10).

Inflammasome recruitment and activation consist of putative sensory molecules (NLRs), adapter proteins (ASC) and Caspase-1 proximal activation leading to inflammatory cytokines and pyroptotic cell death (238). Interestingly, ASC assembly is not always required, as NLRC4 can activate Caspase-1

independently of ASC; however the association of ASC can greatly enhance IL- β production (332). Engaging the inflammasome pathway requires two distinct signals. Cellular sensors such as TLRs and NOD proteins trigger signal 1, resulting in the upregulation of NF- κ B signalling and subsequent increase of pro-IL-1 β and pro-IL-18 transcripts (238). Signal 2, triggered by NLR ligands such as LPS, uric acid, nigericin, involves assembly and activation of the inflammasome complex, leading to cytokine processing and release through pyroptotic cell death (238). Irrespective of inflammatory cytokines, it is perceived that pyroptotic cell death is critical for release of bacteria from their intracellular niche to allow other immune cells to destroy them, contributing to a critical mechanism of immune defence (333).

Inflammasome activation leads to processing and release of pro-inflammatory cytokines such as IL-1 (238). The IL-1 family consists of 11 cytokines that are either inflammatory (i.e. IL-1 α , IL-1 β , IL-18, IL-33) or anti-inflammatory (ie. IL-1Ra) (334). Interestingly, IL-1 cytokines lack signal peptides that prevent them from being secreted by the traditional Golgi transport system (240). Pro-IL-1 α is constitutively expressed as a 31 KDa protein, which can be further processed by calcium dependent proteases known as calpains into a 18KDa protein (335). Pro-IL-1 α is still bioactive and is capable of binding to IL-1R. However, the mature form of IL-1 α has several folds more affinity for its receptor (335, 336). Caspase-1 can also indirectly activate IL-1 α by cleaving IL1R2, an inhibitor of IL-1 α (337). IL-1 β is expressed in its pro-form following NF- κ B signalling and is cleaved by active Caspase-1 following inflammasome

assembly (247, 338, 339). Additional proteases such as proteinase 3 (PR3) and elastases can also cleave IL-1 β (284, 340). Similar to IL-1 β , IL-18 is present in the cell in its pro-form which requires cleavage by caspase-1 mediated inflammasome to be bioactive (341-343). IL-18 activates IFN γ production from cytotoxic CD8⁺ T cells, natural killer (NK) cells and NKT cells (344). Both IL-1 α and IL-1 β bind to IL-1R, while IL-18 binds to the heterodimeric receptor IL-1R1/IL-1RAcP (345). Interestingly, IL-1Ra also binds to IL-1R but acts as an antagonist to limit IL-1 α and IL-1 β signalling and immune activation (334). Anakinra, a synthetic homologue to IL-1Ra is currently being used as a drug to treat inflammatory diseases such as rheumatoid arthritis (346, 347).

1.18 Rationale

Phagosomal pathogens such as Salmonella and Mycobacterium require a CD8⁺ T cell response to effectively eradicate within a given host (348-350). Typically CD8⁺ T cells are activated through proteosomal degradation of antigen present in the cytosol of infected cells (19). However, in cases where the pathogen evades cytosolic residence, the cross-presentation pathway can be employed, allowing for phagosomal pathogens to enter the MHC class-I pathway to induce a proper CD8⁺ T cell response (23). CD8 α DCs are the most relevant subset of DCs that mediate cross-presentation *in-vivo* (101). Therefore, I wanted to address the role of CD8 α DCs during ST infection, and how modulation of both the host (*Nramp1*-dependent innate responses, dependence on Batf3, IFN-I

licencing of DCs) and pathogen (modifying antigen location within infected cells) can influence the adaptive immune response against ST.

Furthermore, many forms of cell death release intracellular contents and activate inflammatory proteins that lead to induction of inflammation (216). Cell death adapters such as caspases and RipKs have been linked to cell death and inflammation (216). A key question in the field is how to uncouple the phenomena of cell death and inflammation and to examine the roles of these death adapter proteins in each context. Many inflammatory receptors also induce activation of cell death pathways, suggesting a strong link between the two distinct pathways (269). I wanted to study how ST evades both the innate and adaptive immune responses by studying the impact cell death and inflammation during infection.

1.19 Purpose

Dr. Sad's laboratory has previously shown that antigen presentation to CD8⁺ T cells, a key mechanism that programs the development of adaptive immunity, is greatly delayed against ST (161). As a result, T cells are non-engaged early on by the time susceptible hosts die, resulting in total immune failure (47, 160, 161). In the absence of early antigen presentation, the host is essentially dependent on innate immunity for protection, and any modulation of innate immunity, such as ageing or pregnancy, can be fatal (351).

1.20 Hypothesis

Since DCs are critical for priming CD8⁺ T cells against intracellular bacteria (which is greatly delayed against ST), I wanted to address how ST modulates key DC functions to curtail the innate and adaptive immune response and maintain a chronic infection, which can result in rapid fatality of mice. I hypothesize that ST delayed a protective CD8⁺ T cell response, by modulating DC functions such as antigen presentation, induction of inflammation and the ability to induce appropriate cell death pathways.

1.21 Objectives

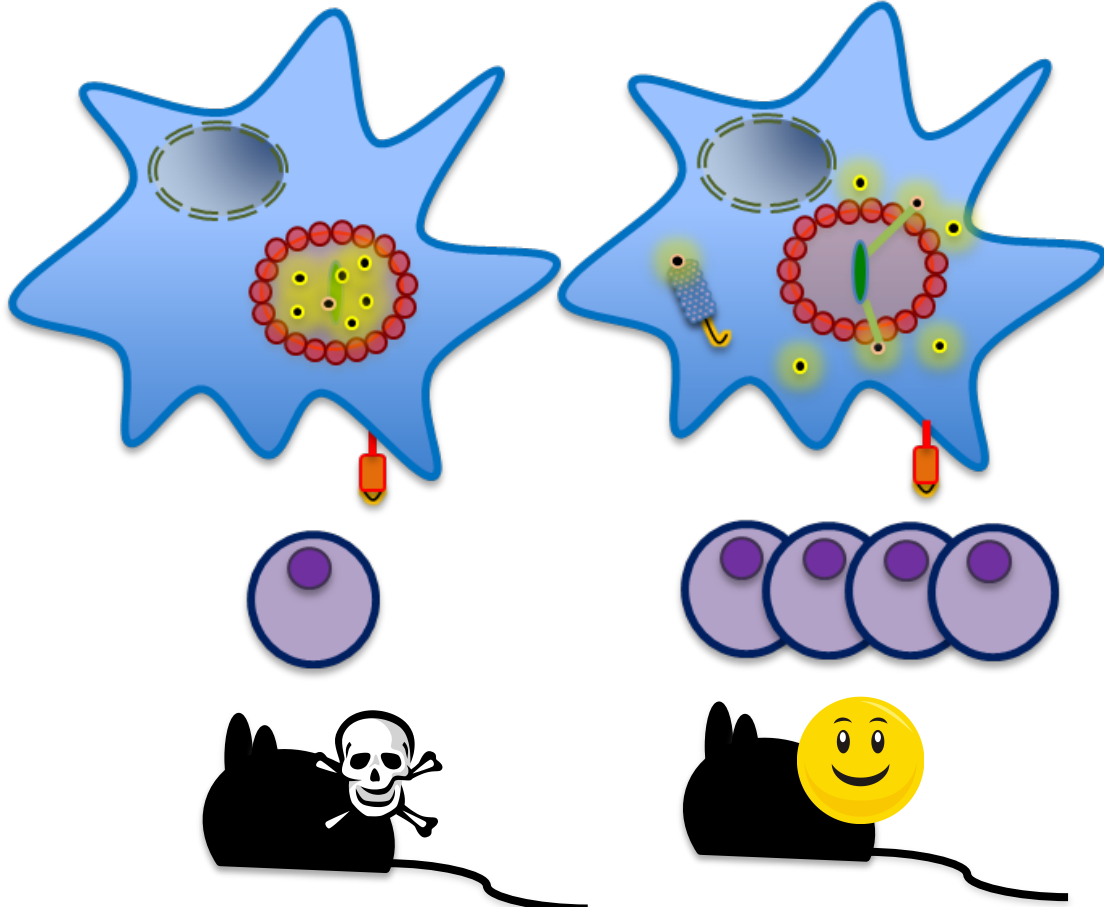
My overall objective is to understand why CD8⁺ T cell priming is critically delayed during infection with ST, and how this response can be facilitated to improve pathogen control. DCs are sentinel immune cells which readily phagocytose ST and are capable of processing phagosomal antigen. This characteristic allows DCs to: (i) initiate a CD8⁺ T cell response critical for immune protection against ST and (ii) mediate innate immune control by engaging pathogen receptors and induce appropriate inflammation and cell death to control ST burden. To address the role of DCs against ST, I modulated the host-pathogen interaction to allow proper assessment of the CD8⁺ T cell response against ST. I have created a transgenic mouse that expresses a functional *Nramp1* gene, which allows for prolonged survival against ST (171, 179). I have also utilized a recombinant strain of ST that deposits model antigen either in the phagosome (STOVA) or cytoplasm (STOVA-C) of infected cells (**Fig. 7**) (46).

Figure 7: Modulation of CD8⁺ T cell response by antigen location

To monitor antigen processing and presentation during an ST infection, we utilized recombinants that express the model antigen ovalbumin (OVA). The recombinant strain ST-OVA maintains deposits OVA antigen in the phagosome of infected cells to induce a delayed CD8⁺ T cell response. In contrast, ST-OVA-C deposits OVA antigen in the cytoplasm of infected cells to induce a rapid CD8⁺ T cell response.

ST-OVA
“Phagosomal”

ST-OVA-C
“Cytoplasmic”



Delayed Response

Rapid Response

1.21.1 Aim1

To determine the role of DC subsets (particularly CD8 α DCs) in mounting a robust CD8⁺ T cell response against systemic ST infection.

1.21.2 Aim2

To evaluate the impact of IFN-I signalling on DC licensing and its impact on priming CD8⁺ T cell response against ST

1.21.3 Aim3

To understand the influence of inflammatory cell death (Caspase-1 mediated pyroptosis and RipK3 mediated necroptosis) on the innate and adaptive immune control of ST.

2: MATERIALS AND METHODS

2.1 Mouse Strains

All mouse strains were bred and maintained in a specific pathogen free facility at the Animal Care and Veterinary Services (ACVS) at Roger Guindon Hall (University of Ottawa, Ottawa, Ontario). All procedures were done in compliance with the Canadian Council on Animal Care (CACC) guidelines. C57BL/6J (stock #000664), 129SvJ (stock #000691), B6.129P2(SJL)-MyD88^{tm1Defr}/J (stock #008888), C57Bl/6-Ticam1^{Lps2}/J (stock #005037), C57Bl/6-Tg(TcraTcrb)1100Mjb/J (stock #003831), 129S-Batf3^{tm1Kmm}/J (stock #013596) and B6.129S(C)-Batf3^{tm1Kmm}/J mice (stock #013755) were purchased from Jackson Laboratory (Bar Harbour, Maine) (**Table 1**). B6.IFNAR1^{-/-} mice were provided by Kaja Murali Krishna (352) (Emory University, Atlanta) (**Table 1**). B6.RipK3^{-/-} mice were provided by Vishva Dixit (Genentech, San Francisco) (**Table 1**) (316). B6.Caspase1/11^{-/-} mice were provided by Richard Flavell (Yale, New Haven) (**Table 1**) (353). B6.Nramp^{G169} mice were provided by Gregory Barton (University of California Berkley) (**Table 1**) (171, 179).

To genotype various mice models, I isolated genomic DNA from ear tissue using the sodium hydroxide (NaOH) method. Ear tissue was collected during

Table 1: Mouse models

This table outlines the various mouse models used to examine the innate and adaptive immune response employed during ST infection.

Mice	Background	Institution	Reference
C57Bl/6	C57Bl/6	JAX	Stock #000664
129SvJ	129Sv/J	JAX	Stock #000691
B6.129P2(SJL)-MyD88 ^{tm1Defr} /J	C57Bl/6	JAX	Stock #008888
C57Bl/6-Ticam1 ^{Lps2} /J	C57Bl/6	JAX	Stock #005037
C57Bl/6-Tg(TcraTcrb)1100Mjb/J	C57Bl/6	JAX	Stock #003831
129S-Batf3 ^{tm1Kmm} /J	129Sv/J	JAX	Stock #013596
B6.129S(C)-Batf3 ^{tm1Kmm} /J	C57Bl/6	JAX	Stock #013755
B6.IFNAR1 ^{-/-}	C57Bl/6	Emory	Dr. Murali Krishna Kaja
B6.RipK3 ^{-/-}	C57Bl/6	Genentech	Dr. Vishva Dixit
B6.Caspase1/11 ^{-/-}	C57Bl/6	Yale	Dr. Richard Flavell
B6.Nramp ^{169G}	C57Bl/6	UC Berkley	Dr. Gregory Barton
B6.Nramp ^{169G} .Caspase1/11 ^{-/-}	C57Bl/6	NRC	Dr. Lakshmi Krishnan / Kristina Wachholz
B6129.F1.N11.IFNAR1	129Sv/J	uOttawa	Dr. Subash Sad / Rajen Patel
B6.Caspase1/11 ^{-/-} RipK3 ^{-/-}	C57Bl/6	uOttawa	Dr. Subash Sad / Bojan Shutinoski
B6.Nramp ^{169G} .RipK3 ^{-/-}	C57Bl/6	uOttawa	Dr. Subash Sad / Rajen Patel
B6.Nramp ^{169G} .Batf3 ^{tm1Kmm} /J	C57Bl/6	uOttawa	Dr. Subash Sad / Rajen Patel

tagging of mice in 1.5mL Eppendorf tubes and treated with 600 μ L of 50mM sodium hydroxide (NaOH) for 20 minutes at 95°C. Samples were mixed by vortexing in 5 minute intervals. Samples were then vortexed for 30 seconds, and neutralized by adding 50 μ L of 1M Tris (pH 7.0), which raises the pH of the sample to 8.3, an ideal pH for polymerase chain reaction (PCR). Samples were centrifuged for 5 minutes and then 400 μ L of the supernatants was transferred to a new tube without disturbing the unwanted pellet.

B6 mice express a non-functional *Nramp1* gene due to a missense mutation that results in a switch from glycine to aspartic acid on the 169th amino acid (in the fourth transmembrane domain) of the Nramp1 protein (179). B6.Nramp mice (homozygous for the functional *Nramp1*^{169G}) were crossed with B6.RipK3^{-/-}, B6. β 2m^{-/-} and B6.Batf3^{-/-} to create the B6.Nramp.RipK3^{-/-}, B6.Nramp. β 2m^{-/-} and B6.Nramp.Batf3^{-/-} mice respectively. Various primer sets were used to verify the expression of RipK3, β 2m and Batf3 (**Table 2**). To determine the expression of *Nramp1*^{169G} in the progeny of different mating scenarios, I isolated DNA from ear tissue and amplified *Nramp1* gene by PCR using a primer set specific for Nramp1 (**Table 2**). PCR products were then sequenced to determine the presence of either homozygous or heterozygous combinations of the 129 allele for Nramp^{G169} or the B6 allele for Nramp^{169D}. Using this approach, B6.Nramp.Caspase1/11^{-/-} mouse strain was created in the lab of Lakshmi Krishnan (National Research Council of Canada, Ottawa, ON). All experiments pertaining to the B6.Nramp.Caspase1/11^{-/-} mice strain were

Table 2: PCR primers

The sequences of various primer sets used to create different mouse models for assessment of immune responses against ST.

Genotyping	Primers	Primer Sequence
<i>β2m</i>	WT primer	5'-CTGAGCTCTGTTTTCTGTCTG-3'
	Common primer	5'-TATCAGTCTCAGTGGGGGTG-3'
	Mutant primer	5'-TCTGGACGAAGAGCATCAGGG-3'
<i>Batf3</i>	WT primer	5'-GTTGTGAGTCGAAACCTCGC-3'
	Common primer	5'-TGCTATGCACAAACCACAAAC-3'
	Mutant primer	5'-CGTTGGCTACCCGTGATATTGC-3'
<i>Nramp1</i>	FW primer	5'-TCATCGGGACGGCTATCTCCT-3'
	RV primer	5'-TTGCGCAAACCTAGGGGTACAGGGA-3'
Caspase1	WT primer	5'-GAGACATATAAGGGAGAAGGG-3'
	Common primer	5'-ATGGCACACCACAGATATCGG-3'
	Mutant primer	5'-TGC AAAGCGCATGCTCCAGACTG-3'
RipK3 WT	FW primer	5'-GGAGCCATTCTCCATGAATC-3'
	RV primer	5'-AATCGTTCCTGGATGGTGAG-3'
RipK3 KO	FW primer	5'-GATCCTGATCCTGACCCTGA-3'
	RV primer	5'-ATCGACAAGACCGGCTTCCATCCGA-3'
<i>IFNAR1 WT</i>	FW primer	5'-TCAGCGCAGGGGCGCCCGTTCTTT-3'
	RV primer	5'-ATCGACAAGACCGGCTTCCATCCGA-3'
<i>IFNAR1 KO</i>	FW primer	5'- AAGATGTGCTGTTCCCTTCTGCTCTG A-3'
	RV primer	5'- ATTATTAAGAAAAGACGAGGCGAAGT GG-3'

performed by Kristina Wachholz (National Research Council of Canada, Ottawa, ON).

B6.Caspase1/11^{-/-} mice were crossed with B6.RipK3^{-/-} mice to create the B6.Caspase1/11^{-/-}RipK3^{-/-} triple knockout mouse strain. To verify that the resultant progeny maintained the disrupted gene of Caspase1, DNA from ear tissue was analysed by PCR for wild type or knockout Caspase1 (**Table 2**). Similarly, DNA from ear tissue was analysed by PCR for wild type RipK3, (**Table 2**) and knockout RipK3 (**Table 2**). The B6.Nramp.RipK3^{-/-} mouse strain was created by crossing B6.Nramp with B6.RipK3^{-/-} mice. The progeny of this mating was screened for the expression of a disrupted *RipK3* gene and *Nramp1* gene using PCR amplification. Nramp1 PCR products were sequenced for the expression of 169G or 169A as described above.

The 129.IFNAR1^{-/-} (B6129.F1.N11.IFNAR1^{neo/neo}) congenic mouse strain expresses a non-functional *IFNAR1* gene disrupted with a Neomycin cassette inserted into exon 2-3 onto the 129SvJ mouse background. This mouse was created by crossing the B6.IFNAR1^{-/-} mouse with the 129SvJ mouse to create a hybrid B6129.F1.IFNAR1^{wt/neo} mouse. These F1 progeny were then crossed back to 129SvJ mouse strain while screening for the mutated IFNAR1^{neo/neo} gene. The resultant progeny was then backcrossed for 11 generations to 129SvJ background. The progeny from the 11th generation was then crossed with each other to create the B6129.F1.N11.IFNAR1^{wt/neo} mouse strain. To ensure that the progeny maintained the disrupted *IFNAR1* gene, DNA from ear tissue was

analysed by PCR for *neomycin cassette*, (**Table 2**). Similarly, DNA from ear tissue was tested for the expression of *IFNAR1^{wt/wt}* (**Table 2**).

2.2 Bacteria and Infections

Salmonella enterica serovar Typhimurium (ST, SL1344), a recombinant of ST that expresses model antigen OVA (ST-OVA); *Listeria monocytogenes* (LM) and a recombinant of LM that expresses OVA (LM-OVA) were all used for *in-vitro* and *in-vivo* infection models. All bacterial strains were used from frozen stock and diluted in 0.9% sodium chloride (NaCl) for *in-vivo* administration. *In-vivo* infections were performed by injecting various doses of bacteria in a total volume of 100µl for intravenous (*i.v.*) injections through the lateral tail vein or 200µL for sub-cutaneous (*s.c.*) injections at the base of the tail. Generation of LM-OVA (354) and ST-OVA (161) has been previously reported. Recombinant strain ST-OVA is hereafter referred to as ST-OVA for the purpose of this publication. Recombinant ST strain (SL1344) that translocates antigen (OVA) from the phagosome to the cytosol is referred to as ST-OVA-C and has been previously described (46). Briefly, truncated OVA protein was incorporated into a plasmid expressed by ST encoding the translocation domain of YopE and the chaperone protein SycE. The fusion protein YopE-OVA is translocated from the phagosome to the cytosol using the type III secretion system of ST, which translocates YopE-OVA protein from the phagosome into the cytoplasm. Mutant strain of ST that lacks expression of flagellin (STΔFlIF) was also used in various studies. Bacterial

burdens were evaluated by plating serial dilutions of spleen and lymph node homogenates onto brain heart infusion (BHI) plates.

2.3 Antibodies and Flow Cytometry

For cell surface staining, anti-CD8 α (53-6-7), anti-CD11b (M170), anti-CD11c (HL3), anti-CD19 (1D3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (42D2), anti-CD205 (205yekta), anti-B220 (RA36B2), anti-TCR β (H57-597), anti-Ly6C (HK1.4), anti-Ly6G (RB6-8C5) and anti-CD49b (DX5) antibodies were purchased from eBioscience (San Diego, CA). Anti-mouse-IA/IE (M5/114.15.2) antibody was purchased from Bio Legend (San Diego, CA). Anti-mouse CD16/CD32 was purchased from BD Bioscience (San Diego, CA). Cell surface staining was performed in phosphate buffered saline (PBS) solution containing 1% BSA at a concentration of 10^6 cells/tube. Cells were treated with anti-mouse CD16/CD32 for 10 minutes at 4°C followed by 0.5 μ L of the surface staining antibodies for 30 minutes at 4°C. H2-K^b-OVA₂₅₇₋₂₆₄ Dextramer (Immudex, Copenhagen, Denmark) staining was performed, for 10 minutes prior to surface staining at room temperature shielded from light.

Anti-CD4 (GK1.5) and anti-CD8 (2.43) antibodies were purified from supernatants of hybridoma cell lines and injected into mice (100 μ g/i.p.) twice a week following infection, to deplete CD4⁺ and CD8⁺ cells respectively. Anti-asialo GM1 from eBioscience (San Diego, CA) was injected into mice (100 μ g/i.p.) twice a week to deplete NK cell populations. Control mice were injected with rat IgG at a similar frequency. Recombinant mouse IL-1 α and IL-1 β were obtained from

R&D Systems (Minneapolis, MN). Recombinant mouse IL-18 was obtained from MBL (Woburn, MA). Recipient mice were injected with either IL-1 α and IL-1 β (100ng/mouse s.c.) or IL-18 (1mg/mouse s.c.) at -1, 0, 1 and 2 days post infection. All recombinant cytokines were diluted in sterile PBS.

2.4 Inhibitors and other Reagents

Various inhibitors were used diluted from stocks in dimethyl sulfoxide (DMSO). Control cells were maintained in media containing comparable amounts of DMSO. The pan-Caspase inhibitor zVAD-fmk was obtained from Calbiochem (San Diego, CA). Caspase-1 inhibitor YVAD was obtained from Calbiochem (San Diego, CA). Caspase-8 inhibitor IETD was obtained from Biovision (San Francisco, CA). RipK1 inhibitor Necrostatin-1 was obtained from Sigma (St Louis, MO). RipK3 inhibitor Debrafenib and SMAC mimetic Birinapant (Bir, cIAP1/2 and XIAP inhibitor) were obtained from Selleckchem (Houston, TX).

2.5 CFSE Staining

Carboxyfluoresceine succinimidyl ester (CFSE) was obtained from Sigma (St Louis, MO). To assess cell proliferation, CD8⁺ T cell were labeled with 0.125 μ M CFSE in PBS at 37 $^{\circ}$ C for 8 min at a concentration of 2x10⁷ splenocytes per mL. The reaction was stopped by adding equal volume of equine serum and placed on ice for 5 minutes, followed by numerous washes with PBS.

2.6 ELISPOT

Enzyme-linked immunospot assays (ELISPOT) were performed to quantify the number of IFN- γ producing cells in various organs harvested from mice challenged with OVA expressing pathogens (161). Cells from either the spleen or draining lymph nodes were isolated from naïve and infected mice at various time points following infection. Cells were enumerated and resuspended in Roswell Park Memorial Institute (RPMI) media-1640 with 8% fetal bovine serum (FBS) known as (R8%) in a Multiscreen IP plate purchased from Millipore (Billerica, MA). Cells were plated at various concentrations of 5×10^5 , 1×10^5 or 2×10^4 cells per well. All wells maintained a total number of 5×10^5 cells, thus the remaining cells were supplemented with naïve feeder cells to maintain a total number of 5×10^5 cells in each well. All cells are treated with the growth factor IL-2 (0.1ng/mL) to promote T cell growth. Wells are designated as either stimulated with peptide (OVA₂₅₇₋₂₆₄, 5 μ g/mL) or incubated with media alone for 48 hours on plates coated with a primary anti-IFN γ antibody (clone R46A2 in NaHCO₃ buffer). Wells were washed with 0.05% PBS-Tween20 buffer and incubated with a biotinylated anti-IFN γ antibody (clone XMG1.2) for two hours. Following a few stringent washes, wells were washed and incubated with streptavidin-horse radish peroxidase (HRP) from Sigma (St. Louis, MO) for 1 hour. After HRP treatment, wells were washed and incubated with 3-amino-9-ethylcarbazole (AEC) substrate to quantify IFN γ producing spots in each well.

2.7 *In-vivo* CTL

In-vivo cytotoxic T lymphocyte (CTL) assays were used to assess target cell killing by endogenous CD8⁺ T cells within a given infected mouse. Briefly, splenocytes from syngeneic naïve mice were harvested and divided into two groups: naïve (non-target) cells and antigen coated (target) cells. The model antigen utilized was OVA. Target cells were resuspended to 10 million cells/mL and treated with OVA₂₅₇₋₂₆₄ peptide at 10mg/mL in R8% media for 30 minutes. This procedure allows for external peptide loading of OVA₂₅₇₋₂₆₄ peptide onto MHC class-I molecules, making them targets for OVA₂₅₇₋₂₆₄ specific CD8⁺ T cells. As a control, naïve (non-target) cells were incubated in R8% media alone for 30 minutes at a similar cell concentration of 10 million cells/mL. Both target and non-target cells were then washed, resuspended to 20 million cells/mL and labeled with either 2mM or 0.2mM of CFSE respectively for 30 minutes. CFSE labeling was stopped by treating cells with equine serum on ice for 5 minutes. Target and non-target cells were resuspended to a final concentration of 100 million cells/mL in Hank's balanced salt solution (HBSS) and mixed in a ratio of 1:1. A total volume of 200µL of target/non target cells were injected into each recipient mouse (*i.v.*), which are either naïve or previously infected with bacteria expressing OVA (ie. ST-OVA-P, ST-OVA-C, LM-OVA) to induce a cytotoxic CD8⁺ T cell response. Sixteen hours following injection of target and non-target cells, mice were sacrificed; spleens and lymph nodes were collected and assessed for CFSE^{hi} target and CFSE^{lo} target cells by flow cytometry. Percent killing of target cells was calculated as follows:

$$CTL \text{ killing} = 100\% - 100 \times \frac{\left(\frac{\%target \text{ killing}}{\%non \text{ target killing}} \right)_{infected \text{ mice}}}{\left(\frac{\%target \text{ killing}}{\%non \text{ target killing}} \right)_{naive \text{ mice}}}$$

2.8 Adoptive Transfer

CD8⁺ OT-1 cells were purified from spleens harvested from OT-1 mice (CD45.1⁺CD45.2⁺) using a CD8⁺ negative selection kit from Stemcell Technologies (Vancouver, BC). OT-1 CD8 T cells express a transgenic TCR that is specific for H2-K^b-OVA₂₅₇₋₂₆₄ and thus provides an ideal model for tracking pathogens expressing the model antigen OVA (355, 356). One day prior to bacterial infection, 10⁵ CD8⁺ OT-1 cells were injected (resuspended in 200µL *i.v.*) into recipient mice (CD45.1⁻CD45.2⁺). Blood samples were collected using saphenous bleeding at different time points post infection and treated with ammonium-chloride-potassium (ACK) lysis buffer from Sigma (St. Louis, MO). To determine the number of adoptively transferred OT-1 CD8⁺ T cells present in each sample, cells were stained for CD45.1 and CD45.2 and assessed using flow cytometry to determine relative percentages of endogenous CD8⁺ T cells (CD45.1⁻CD45.2⁺) and adoptively transferred OT-1 CD8⁺ T cells (CD45.1⁺CD45.2⁺).

2.9 Isolation of DCs and other cells

To isolate cells from spleen and draining lymph nodes, organs were harvested and mechanically disrupted using frosted glass slides. To isolate leukocytes from the spleen, cells were treated with ACK lysis buffer for 1 minute,

followed by washing with R8% media. To isolate cells from lymph nodes, homogenates were spun down and supernatant containing any fat was removed. Finally, cells were strained using a 70µm cell strainer and used for experiments.

To isolate DC subsets, spleens were harvested, mechanically dissociated using a sterile blade and treated with dissociation media (Stemcell, Vancouver, Canada) for 30 minutes. CD11c cells were purified using a PE-CD11c positive selection kit (Stemcell). Briefly, cells were treated with FcR blocker, CD11c PE labeling reagent, PE selection cocktail and magnetic nanoparticles as per manufacturer's protocol. Cells of interest were purified using magnetic isolation as CD11c cells labeled with PE antibodies bound to magnetic particles through positive selection. Cells were verified to be ~90% positive for CD11c using a CyanADP flow cytometry analyzer (Beckman Coulter, Mississauga, Ontario). Cells were also stained for additional surface markers to characterize DC subsets such as CD8α DCs (B220⁻CD11c⁺MHCII⁺CD11b⁻CD8α⁺), CD11b DCs (B220⁻CD11c⁺MHCII⁺CD11b⁺CD8α⁻) and pDCs (B220^{lo/+}CD11c⁺). Cells were also sorted for different DC populations using the MoFlo Astrios Sorter (Beckman Coulter, Mississauga, Ontario) and assessed for purity by flow cytometry.

2.10 Bone Marrow Derived Macrophages and DCs

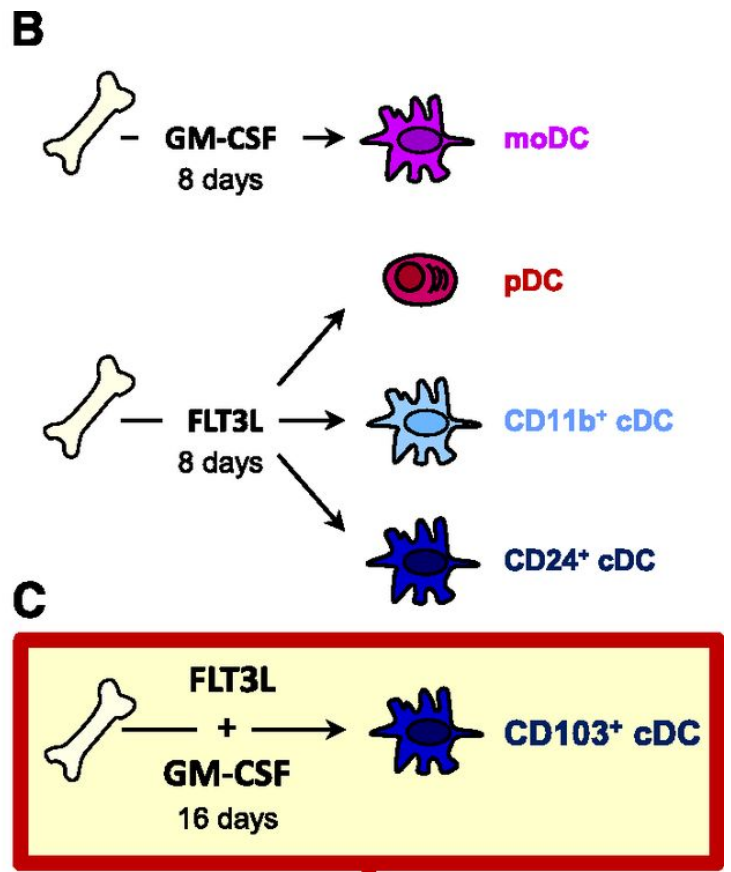
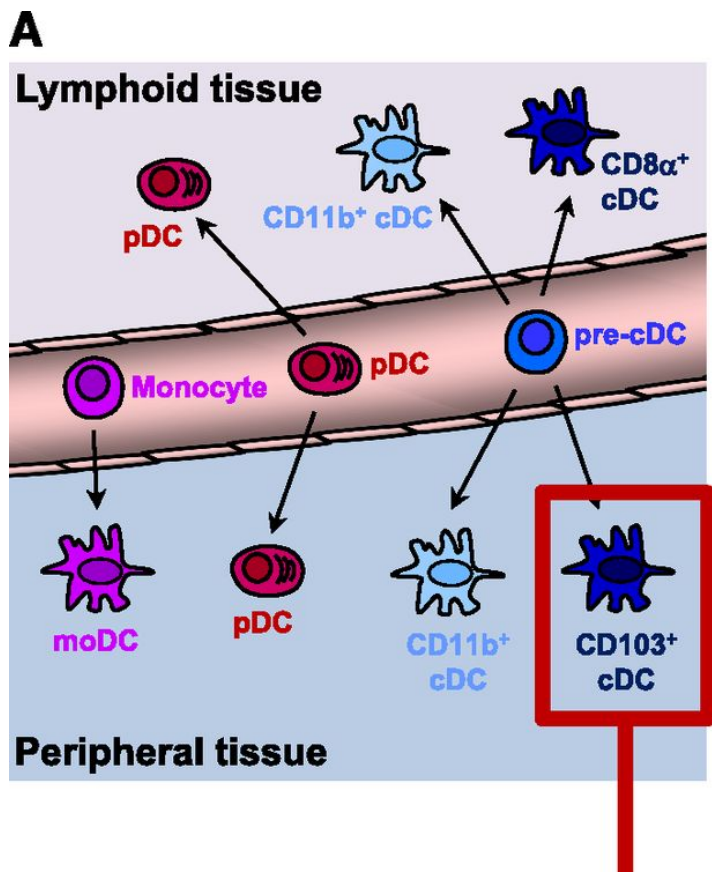
Bone marrow DC (BMDC) cell cultures were grown *in-vitro* using bone marrow progenitors and various growth factors to mimic DC populations found *in-vivo*. Bone marrow derived dendritic cells grown with GM-CSF (GMCSF-DC), which mimic MoDCs (357-359), were grown by culturing bone marrow progenitors isolated from the femur and tibia of representative mouse strains

(360, 361). Cells were cultured at a concentration of 2 million cells/mL in R8% medium in the presence of the growth factor GM-CSF from Empire Genomics (Buffalo, NY) at a concentration of 5ng/mL for 7-8 days in T25 flasks at 37°C. Media was replaced at day 2 and 4 to remove any floating cells. By day 7-8, GMCSF-DCs differentiate from adherent cells (macrophages) and become floating cells that can be collected from the supernatant (**Fig. 8**). These cells were collected at this time and used for experiments. To grow FLT3L-derived DCs (362), bone marrow progenitors were cultured with FLT3L (300ng/mL) in 6 well plates at a concentration of 2 million cells/mL. Following 9-12 days of incubation at 37°C, cells differentiate into CD8 α DCs (362). Interestingly, these cells do not express CD8 α but express other cell surface markers (ie. CD24) and have functional characteristics related to CD8 α DCs found *in-vivo* (**Fig. 8**) (362). To grow induced CD103⁺ DCs *in-vitro* (CD103i DCs) (363), bone marrow progenitor cells were cultured at a concentration of 2 million cells/mL with FLT3L (50ng/mL) and GM-CSF (2ng/mL) in petri dishes at 37°C. Following 5-6 days of incubation, 5mL of fresh R8% medium was added to each petri dish to prevent cell death. At day 9, cells were harvested and resuspended at a concentration of 3 million cells/mL in petri dishes with additional growth factors (50ng/mL FLT3L and 2ng/mL GM-CSF). Cells were grown at 37°C for an additional 7 days (for a total of day 16). At this point, cells should be floating and clustering with many foci and resemble CD103⁺ DCs found *in-vivo* (**Fig. 8**). To culture BMMs, bone marrow progenitors were isolated from the femur and tibia of respective mouse strains. Cells were counted, resuspended at 1 million cells/mL and grown in R8%

Figure 8: *In-vitro* cultures of dendritic cells

DCs can be cultured *in-vitro* with the use of various growth factors such as FLT3L and GM-CSF. **(A)** Five major subsets of DCs can be grown *in-vitro*: pDCs, CD11b DCs, CD8 α DCs, CD103⁺ DCs (which are all found in the lymphoid and peripheral tissue *in-vivo*) and moDCs (which are monocyte derived DCs under inflammatory conditions such as infection found exclusively in the periphery). **(B)** Bone marrow cultures grown in the presence of GM-CSF yields *in-vitro* moDC equivalents called GMCSF-DCs. Bone marrow cells incubated with FLT3L can result in the differentiation of CD24 (CD8 α equivalent DCs), CD11b DCs and pDC, generally termed FLT3L-DCs. Finally, bone marrow progenitors grown in the presence of FLT3L and GM-CSF can yield CD103ⁱ DCs (CD103⁺ DC equivalents).

Figure as originally published in van de Laar L and Lambecht BN (2014) How to generate large numbers of CD103⁺ dendritic cells. Blood 124:20 doi: 10.1182/blood-2014-08-595298.



in the presence of the growth factor M-CSF (5ng/mL) from R&D Systems (Minneapolis, MN) for 6 days (**Fig. 8**).

2.11 *In-vitro* Infection Assay

For *in-vitro* direct presentation assays, spleens were harvested from mice, treated with dissociation media from Stemcell (Vancouver, BC) for 30 minutes and purified for CD11c DCs cells using a PE-CD11c positive selection kit (Stemcell). Cells were plated at 5×10^4 cells per well in a round bottom 96-well plate in R8%. Cells were infected with bacteria for 30 min followed by three washes and a two hour treatment with high gentamycin (50 μ g/ml). These cells were then incubated with 5×10^4 CFSE labeled, OT-1 CD8⁺ T cells (purified using a negative selection kit) in gentamycin containing R8% medium (10mg/mL).

For *in-vitro* cross-presentation assays, splenocytes were harvested from mice, treated with dissociation media and purified for CD11c DCs cells using a PE-CD11c positive selection kit. Cells were plated at 5×10^4 cells per well in a round bottom 96-well plate in R8% and infected with bacteria for 30 minutes in the presence of β 2m-deficient splenocytes loaded with OVA protein through osmotic shock (101). Briefly, cells were incubated in a hypertonic medium (0.5M sucrose, 10% wt/vol polyethylene glycol 1000 and 10mM HEPES in RPMI) containing OVA protein for 10 minutes. This allows for loading of OVA into β 2m^{-/-} splenocytes through osmotic pressure. Cells were then incubated in a hypotonic solution (40% H₂O and 60% RPMI) for two minutes to recuperate from the previous treatment. Cells were then treated with 1350 RAD of radiation to prevent

growth of $\beta 2m^{-/}$ splenocytes following incubation with CD11c DCs. Cells were washed and treated for two hours with gentamycin (50 μ g/ml). These cells were then incubated with 5×10^4 CFSE labeled, purified OT-1 CD8⁺ T cells using a negative selection kit in gentamycin treated R8% media (10mg/mL). Following 72 hours of incubation, CFSE dilution of OT-1 CD8⁺ T cells is assessed using flow cytometry. Since the $\beta 2m$ -deficient splenocytes do not express an MHC-I molecules on the surface of the cell, the only way OVA can be presented to activate OT-1 CD8⁺ T cells is if CD11c DCs acquire OVA loaded $\beta 2m$ splenocytes during ST infection through phagocytosis and cross-present the cellular antigens onto MHC-I.

2.12 LPS Treatment

For *in vitro* experiments, cells were plated into 96 well plates and treated with 100ng/mL of LPS from Sigma (St. Louis, MO) for 24 hours. Supernatants were collected and assessed for cytokine and chemokine production. For *in-vivo* experiments, mice were injected (*i.p.*) with 50 mg/kg of LPS from Sigma (St. Louis, MO). Mice were monitored for general health using body temperature measurements. Generally, mice were euthanized at 6 hours following LPS injections and serum samples were collected for cytokine and chemokine analysis.

2.13 Cell Death Assays

Cell death was measured using neutral red uptake assay. Cells were plated into 96 well plates at a concentration of 10^5 cells per well. Following various

stimulations or infections, neutral red dye was added to each well at a concentration of 0.17g/mL diluted in R8% media for 5-10 minutes at 37°C. Once cells had taken up the dye and were visibly red, (determined by viewing under the light microscope), all wells were washed with PBS. Solubilization solution (50% ethanol, Et-OH; 50% water, H₂O and 1mL glacial acetic acid, CH₃COOH) was added to each well. Absorbance was measured at 570-650nm using the FilterMax F5 microplate reader from Molecular Devices (Sunnyvale, CA).

2.14 Cytokine and Chemokine Quantification

Supernatants from *in-vitro* experiments and serum samples from *in-vivo* experiments were collected and analyzed for the presence of cytokines and chemokines. Cytokines IL-1 α , IL-1 β , IL-6, TNF, IL-10 and the chemokines macrophage inflammatory protein (MIP)-1 α , MIP-1 β , regulated on activation normal T cell expressed and secreted (RANTES), keratinocyte-derived cytokine (KC), monocyte induced by gamma interferon (MIG) and monocyte chemoattractant protein-1 (MCP-1) were analyzed using cytokine bead array (CBA) kits from BD Bioscience (San Diego, CA). In some cases, expression of cytokines and chemokines were detected using a Mouse Proteome Array kit from R&D Systems (Minneapolis, MN). Expression of cytokines/chemokines in the proteome array was quantitated by chemiluminescence detected using a Fluorochem 8900 imager (Alpha Innotech). Densitometric expression values were enumerated using either AlphaEase software and were corrected to the

internal positive controls and expressed as mean fold change over uninfected samples.

2.15 Histology

Spleens harvested from infected mice were fixed in neutral buffered 10% formalin for 48 hours followed by treatment in 70% ethanol. Spleens were then embedded in paraffin and vertical sections were stained with hematoxylin and eosin (H&E) to assess pathology. Histology sections were scored by Dr. Manijeh Daneshmand from the Pathology Department at the University of Ottawa.

2.16 Western Blot

Samples were lysed in sodium dodecyl sulfate (SDS) β -mercaptoethanol (β -ME) buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Samples were transferred onto polyvinylidene fluoride (PVDF) membranes, probed with antibodies of interest and developed using enhanced chemiluminescent (ECL) or Femto substrates from Life Technologies (Carlsbad, CA) on X-ray films. The following antibodies were obtained from Cell Signaling Tech. (Beverly, MA): Rabbit anti-p65 NF- κ B (D14E12), rabbit anti-pp65 NF- κ B (93H1), rabbit anti-p38 MAPK (D13E1), and rabbit anti-pp38 MAPK (T180/Y182). Additional antibodies were purchased from the following sources: Mouse anti-Rip1 (38/RIP) from BD Biosciences (San Jose, CA), Rat anti-Caspase-8 (1G12) from Enzo (Farmingdale, NY), Rabbit anti-Ipaf/NLRC4 (06-1125) from EMD Milipore (Billerica, MA), Rat anti-NLRP3 (768319) from R&D System, (Minneapolis, MN), Rabbit anti-IL-18 (5180R-100) from Biovision (Milpitas, CA),

Mouse anti- β -actin (ACTBD11B7) from Santa Cruz (Dallas, TX), Rabbit anti-RipK3 (2283) from ProSci (Poway, CA) and Rabbit anti-clAP1/2 (CY-P1041) from Cyclex (Nagano, Japan). Densitometric expression values were enumerated using either ImageJ software by comparing the expression of the house keeping gene β -actin to the expression of various proteins. Densitometric values were calculated as a ratio of expression of the target protein expression over the expression of β -actin.

2.17 Statistical Analysis

Statistics were done using GraphPad Prism (GraphPad Software, California, USA). In general, statistical significance was determined by unpaired student t-test. The statistical significance of survival data was evaluated by Gehan-Breslow-Wilcoxon test.

3: RESULTS

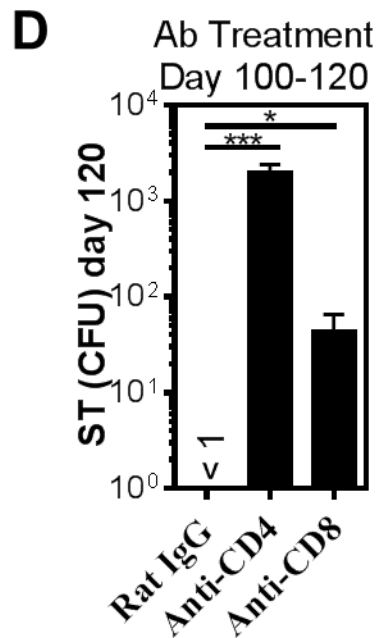
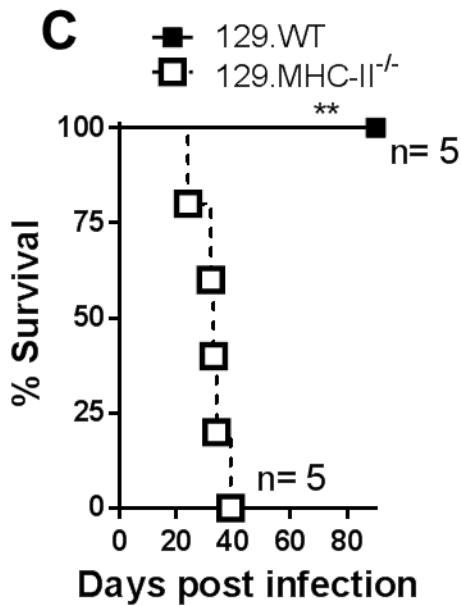
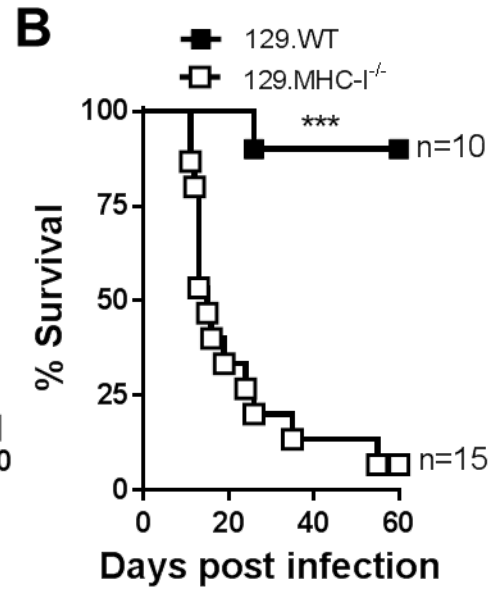
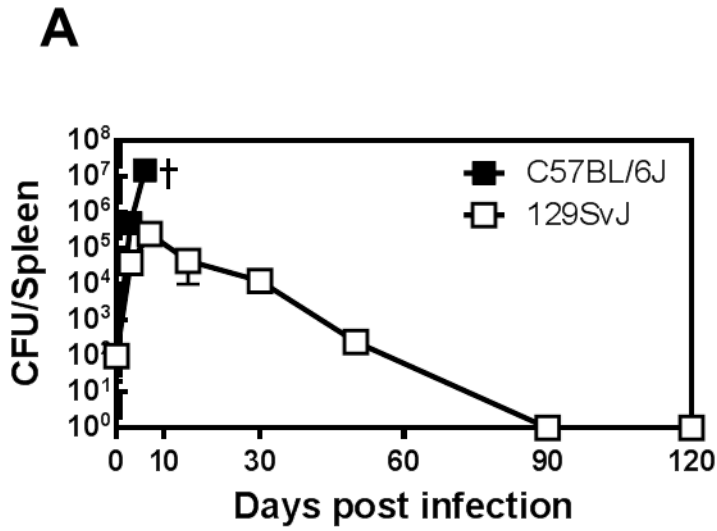
3.1 CHAPTER 1: IFN-I signalling is critical to mount a CD8⁺ T cell response against ST

3.1.1 CD8⁺ T cells mediate immune protection against ST

C57BL/6J (B6) and 129SvJ (129) mice represent two inbred strains of mice that have varying immune defenses against ST, which greatly impacts host survival (160, 161). To validate my model, I first infected susceptible B6 and resistant 129 mice with virulent ST (10^2 CFU *i.v.*) strain SL1344, and assessed bacterial burden in the spleens at various time points following infection. As previously reported, B6 mice succumb to infection at an early stage (within 10 days) (161). In stark contrast, 129 mice maintain a chronic infection that lasts about 120 days; at which point ST is no longer detectable in the spleen (**Fig. 9A**) (161). CD4⁺ T cells have been considered to be critical cell subset that mediates protection against ST (364-366), and the role of CD8⁺ T cells has been unclear. Since MHC class-I deficient mice are available only on B6 background, the role of CD8⁺ T cells against virulent ST has not been evaluated due to premature death of B6 mice. Thus, I generated $\beta 2m$ -deficient mice onto the 129 background and tested their survival against ST infection. I infected MHC class-I deficient

Figure 9: CD4⁺ and CD8⁺ T cells mediate control of chronic ST in resistant mice

(A) Susceptible B6 and resistant 129 mice were infected with ST (10^2 *i.v.*) and assessed for bacterial burden at various time points following infection with ST. Mice were sacrificed at various time points, spleens were harvested and assessed for bacterial burden by serial dilution onto brain heart infusion (BHI) plates. **(B)** MHC class-I deficient (B6.129. $\beta 2m^{-/-}$) mice and wild-type littermates were infected with ST (10^3 *i.v.*) and assessed for survival. **(C)** MHC class-II deficient (H2^{dIAb1-Ea^{-/-}}) mice and wild-type controls, both on the 129 strain, were infected with ST (10^3 *i.v.*) and assessed for survival. **(D)** Resistant 129 mice were infected with ST (10^2 *i.v.*) and treated with depleting anti-CD4, anti-CD8 and control IgG antibodies during the late (day 100-120) stage of infection. Bacterial burdens were assessed at day 20 (for early stage depletion) and day 120 (for late stage depletion) by serial dilution on BHI plates. Representative data is shown as mean \pm SEM from 2-3 experiments. Experiments examining bacterial burden utilize a minimum of three mice per group. All data points represent an n=3 unless otherwise stated. Statistical significance on survival data was determined by the Gehan-Breslow-Wilcoxon test (**p<0.01, ***p<0.001).

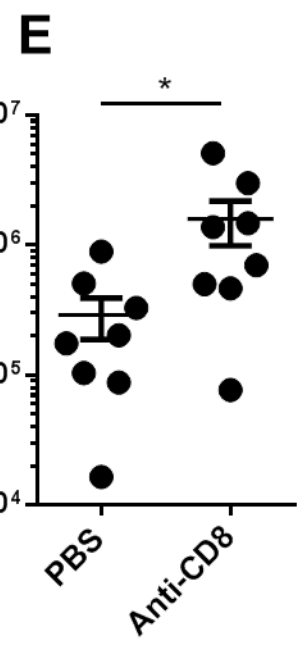
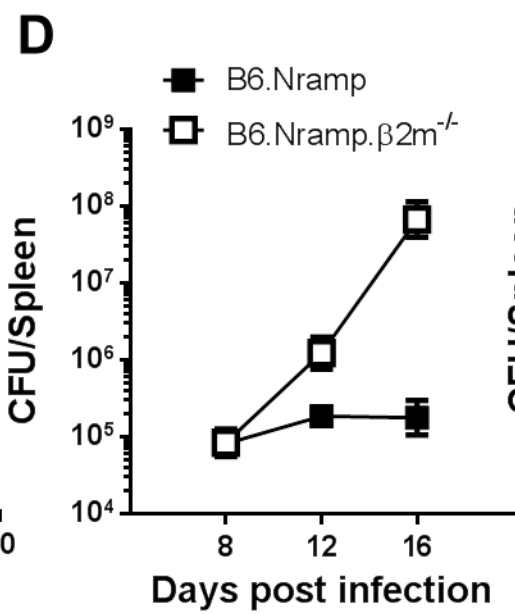
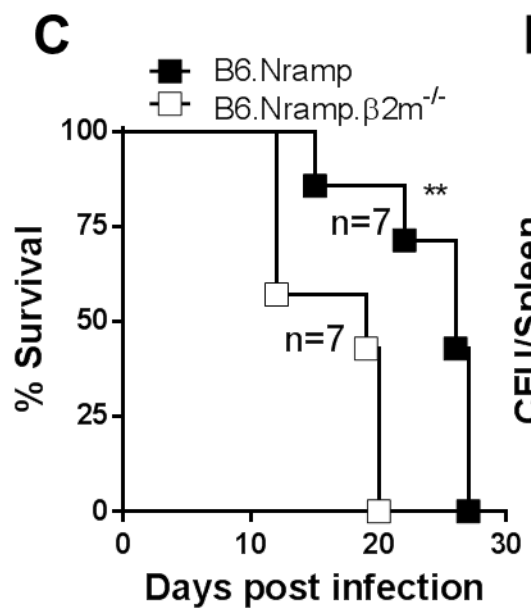
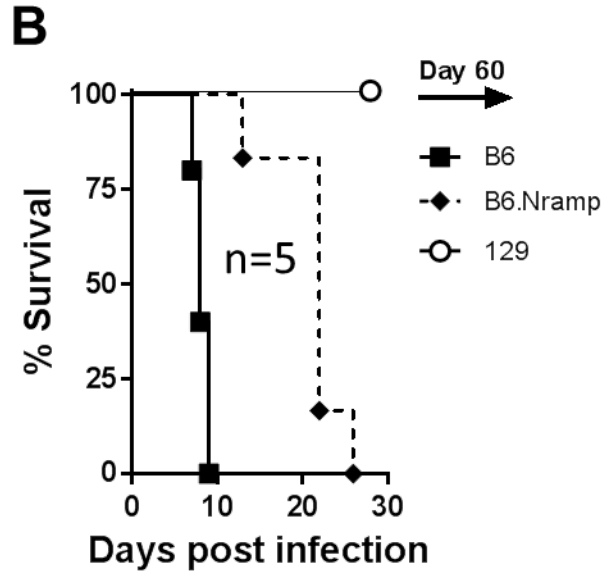
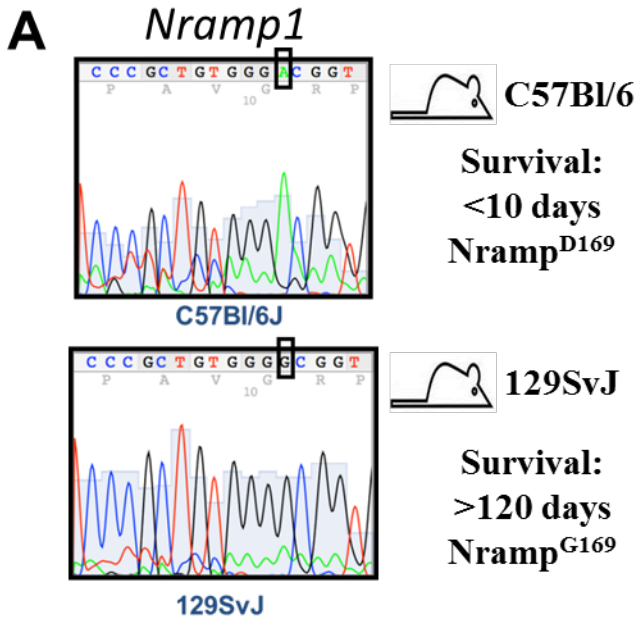


(F3.B6129. β 2m^{-/-}) mice, which effectively lack any CD8⁺ T cells (367), with ST (10³ CFU *i.v.*) to test their survival following systemic infection. While majority of WT mice survived ST infection, MHC class-I deficient mice displayed poor resistance to infection (**Fig. 9B**). MHC class II-deficient mice were available on the 129 background, and I therefore tested the role of CD4⁺ T cells against infection with virulent ST. MHC class II-deficient displayed poor survival following infection with ST (**Fig. 9C**). Decreased host survival can be attributed to the fact that MHC class II-deficient mice are devoid of CD4⁺ T cells (368), which can also impact the humoral immune and provide help to the CD8⁺ T cell response. To specifically evaluate the role of CD4⁺ versus CD8⁺ T cells during the late stage of infection, when the bacterial burden is undetectable, I depleted CD4⁺ versus CD8⁺ T cells with antibodies from day 100 to day 120, and measured the impact on bacterial burden at day 120 post-infection. Control mice that were treated with isotype IgG antibody had no detectable bacterial burden at day 120 following infection (**Fig. 9D**). In contrast, treating mice with anti-CD4 and anti-CD8 depleting antibody result in an increase in splenic bacterial burden at late stages of infection (**Fig. 9D**). These results indicate that both of these subsets mediate immune surveillance against ST and depletion of these cells results in resurgence of bacteria. Furthermore, it appeared that CD4⁺ T cells play a more protective role in comparison to CD8⁺ T cells. However, my data suggests that the depletion of CD4⁺ T cells also compromises the CD8⁺ T cell response against ST (**unpublished data**).

B6 mice succumb to virulent ST infection at early stages of infection. Consequently, the role of CD8⁺ T cells in the context of ST infection in B6 mice has been evaluated only in attenuated mutants of ST (lack key virulence factors) or auxotrophic strains of ST (lacking metabolic genes) that do not induce a fatal infection in susceptible mice (349, 366, 369). Thus the role of CD8⁺ T cells against ST in susceptible hosts remains to be elucidated with relevant virulent (pathogenic) strains of ST, which can impact host survival and provide insight into immune evasion. *Nramp1* is a critical innate resistance gene that promotes immune defense against ST and other intracellular pathogens (179, 370). It encodes a membrane protein that allows efflux of divalent ions out of the phagosomal lumen, contributing to the harsh anti-microbial environment in the phagosome resulting in pathogen control (179). B6 mice express a mutation in the *Nramp1* gene (*Nramp1*^{D169}), which contributes to host susceptibility against ST. In contrast, 129 mice express a functional *Nramp1* gene (*Nramp1*^{G169}) which results in host resistance to ST infection (**Fig. 10A**) (178, 179, 183). Following infection with ST, B6 mice succumbed to infection rapidly, in contrast to the 129 strain that controlled the chronic infection and survived past 4 months (**Fig. 10B**). I used B6 mice expressing transgenic wild-type *Nramp1* gene (henceforth referred to as B6.Nramp mice) and noted enhanced survival in comparison to wild type mice (**Fig. 10B**). However, all mice succumbed to infection by day 30, implying that the resistance of the 129 strain must involve additional genetic factors besides the functional *Nramp1* gene (185).

Figure 10: CD8⁺ T cells mediate immune control of ST in Nramp1 expressing mice

(A) Inbred mouse strains express different polymorphisms of the *Nramp1* gene. Susceptible B6 mice express the polymorphism Nramp^{D169} resulting in a non functional Nramp1 protein (resistant mice maintain a chronic infection with prolonged host survival >120 days). In contrast, resistant 129 mice express the polymorphism Nramp^{G169} resulting in a functional Nramp1 protein (susceptible mice die rapidly following infection within 10 days). **(B)** B6, B6.Nramp and 129SvJ mice were challenged with ST (10^3 *i.v.*) and assessed for host survival. B6.Nramp and B6.Nramp.β2m^{-/-} were infected with ST (10^3 *i.v.*) and assessed for **(C)** host survival and **(D)** splenic bacterial burden. **(E)** To deplete CD8⁺ cells, B6.Nramp mice were administered with anti-CD8 depleting antibody (clone 2.43) two days before infection twice a week for two weeks. Mice were infected with ST (10^3 *i.v.*) and assessed for splenic bacterial burden at day 13. Representative data is shown as mean ±SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance on survival data was determined using Gehan-Breslow-Wilcoxon tests (*p<0.05, **p<0.01).



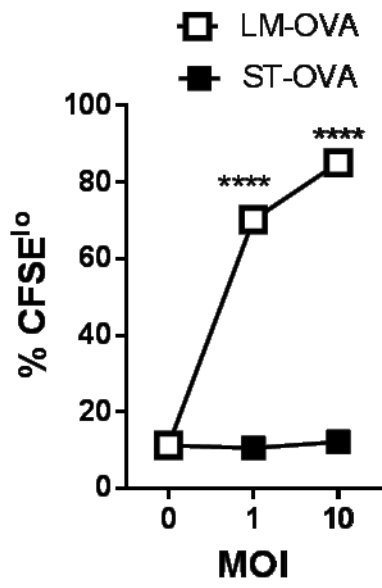
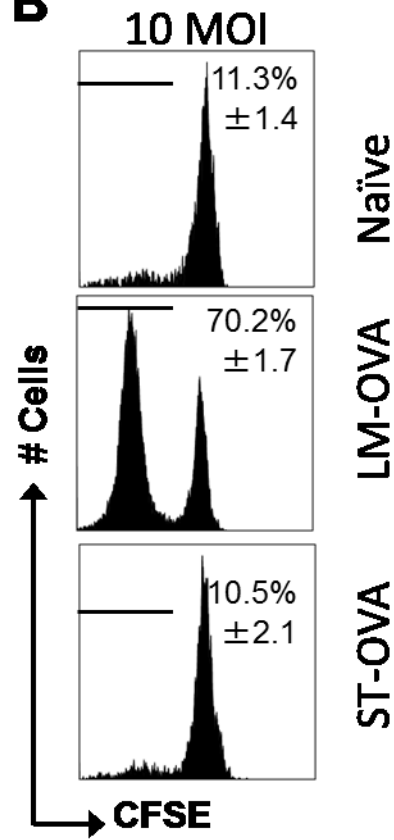
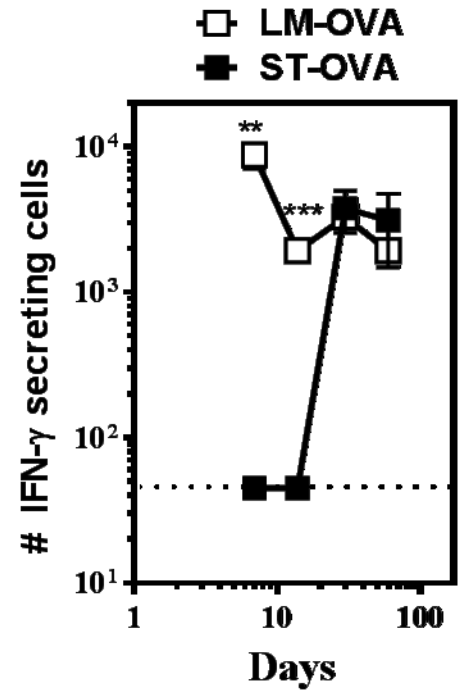
Prolonging the survival of B6 against ST in the B6.Nramp mouse model (>20 days) allowed us to evaluate the impact of CD8⁺ T cells against ST on the susceptible background. Accordingly, I generated MHC class-I deficient ($\beta 2m^{-/-}$) mice on the B6.Nramp background to evaluate the impact of CD8⁺ T cell responses against ST during the first few weeks of infection. I noted that MHC class-I deficient mice were more susceptible to ST infection compared to their wild type counterparts, as shown by decreased survival (**Fig. 10C**) and enhanced bacterial burden (**Fig. 10D**). Additionally, depletion of CD8⁺ cells (most likely CD8⁺ T cells) in B6.Nramp mice following administration of anti-CD8 depleting antibody resulted in a significantly higher bacterial burden at early stages of infection (**Fig. 10E**). Taken together, these results indicate that CD8⁺ T cell response against virulent ST is important in both resistant and susceptible strains of mice at early and later stages of infection.

3.1.2 ST infected DCs fail to mediate early antigen presentation

Host cells infected with the cytosolic pathogens such as LM induce early antigen presentation, which results in rapid development of a CD8⁺ T cell response against these pathogens (35, 39, 40). Pathogen derived antigens expressed in the cytosol are readily degraded by the proteasome and processed by the classical MHC class-I pathway to activate CD8⁺ T cells (96, 371, 372). Infection of splenic DCs with recombinant ST expressing the model antigen OVA failed to induce any proliferation of CFSE-labeled CD8⁺ T cells from OT-1 mice (expressing a transgenic TCR specific for OVA₂₅₇₋₂₆₄ peptide) (**Fig. 11A-B**). In

Figure 11: Rapid antigen-presentation is not induced by DCs infected with ST

(A) To measure the differential capability of direct-presentation of different intracellular bacteria, 5×10^4 B6 CD11c cells (purified by CD11c⁺ Stemcell kit) were plated into a 96 well plate and infected with LM-OVA or ST-OVA for 30 minutes. Following infection, cells were washed three times with R8 media and incubated for 2h with gentamycin (50ug/ml) to eliminate any extracellular bacteria. CFSE labelled CD8⁺ T cells isolated from OT-1 mice were added and the CFSE dilution was assessed after 72h as an indication of proliferation. **(B)** Representative FACS plot of CFSE dilution in OT-1 cells cultured at an MOI of 10 is shown. **(C)** B6129F1 mice were infected intravenously with LM-OVA or ST-OVA (10^3 CFU). At various time intervals, frequency of IFN- γ ⁺ OVA-specific CD8⁺ T cells was enumerated by ELISPOT assay from spleens harvested from infected mice. Representative data of 2 experiments is shown. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (**p<0.01, ***p<0.001, ****p<0.0001).

A**B****C**

contrast, infection of DCs with the cytosolic pathogen, LM-OVA resulted in rapid antigen presentation (**Fig. 11A-B**). This stark difference in antigen presentation between LM and ST could be attributed to their intracellular lifestyle. The antigens of LM are available in the cytosol for degradation by the proteasome, whereas ST derived antigens are sequestered in the phagosome (46, 154, 373-375). I infected 129SvJ mice with LM-OVA and ST-OVA and noted that there was a significant delay in the CD8⁺ T cell response against ST-OVA (**Fig. 11C**). In contrast, the response against LM-OVA peaked within 7 days of infection (**Fig. 11C**). However, the response that was induced later on against ST-OVA was potent in magnitude, presumably by the cross-presentation pathway (**Fig. 11C**).

3.1.3 Poor CD8⁺ T cell response in IFNAR1-deficient mice following ST infection

IFN-I signalling has been shown to be critical for many aspects of innate and adaptive immunity to promote protection against viral infections (186). More recently, the impact of IFN-I signalling has also been described to encompass aspects of bacterial immunity, induction of cell death, antigen processing and antigen presentation (111, 193, 207, 231). In particular, IFN-I signalling has been shown to dictate cross-presentation by modulating antigen processing in the phagosome (111, 208, 213). Cross-presentation of cellular antigen can be measured *in-vitro* by loading β 2m-deficient splenocytes with OVA protein and incubating these cells with splenic CD11c DCs. CD8⁺ T cell priming of CFSE labelled CD8⁺ OT-1 T cells can be measured by monitoring CFSE dilution (T cell proliferation). This is indicative of cross-presentation of cellular OVA antigen. I

utilized this experimental protocol to confirm the impact of IFNAR signalling on cross-presentation of cellular antigen by showing that IFNAR1^{-/-} DCs have a diminished capacity to cross-present OVA antigen in comparison to the wild type DCs (**Fig. 12A**). Furthermore, treatment with TLR agonists augmented the cross-presentation of cell associated OVA (**Fig. 12B**). Infection with pathogenic bacteria induces pro-inflammatory cytokines, which can impact antigen presentation. To examine the impact of IFNAR signalling on cross-presentation in the context of ST infection, I incubated OVA loaded β 2m-deficient cells with wild type and IFNAR1-deficient CD11c DCs infected with ST (**Fig 12C**). Even in this model, IFNAR signalling promoted cross-presentation of OVA. Furthermore, MyD88 signalling was critical in mediating ST-induced cross-presentation (**Fig. 12D**).

I then tested the impact of IFNAR signalling on CD8⁺ T cell priming *in-vivo*. I used a localized infection model, where ST was subcutaneously administered at the base of the tail along with soluble OVA. As sentinel immune cells such as DCs acquire a combination of ST and OVA in the phagosome, they can induce cross-presentation of OVA protein in the context of the inflammatory response mediated by ST. I measured the CD8⁺ T cell response against OVA in the draining inguinal lymph nodes using the *in-vivo* CTL killing assay, where endogenous OVA₂₅₇₋₂₆₄ specific CD8⁺ T cell mediated elimination of target cells was measured. I observed killing of OVA₂₅₇₋₂₆₄ pulsed target cell in wild type mice (**Fig. 13**). In contrast, the IFNAR1-deficient mice had a greatly diminished CTL killing of target cells, indicating CD8⁺ T cell priming was defective in IFNAR1-

Figure 12: Cross-presentation during ST infection is partially dependent on IFN-I during inflammatory conditions induced by ST

(A) Wild type and IFNAR1^{-/-} DCs purified from splenocytes were plated onto 96 well plates, and co-incubated with β 2m cells loaded with OVA protein for 30 minutes. Cells were then washed and incubated with CFSE labeled OT-1 CD8⁺ T cells for 72 hours and assessed for proliferation (CFSE dilution) by flow cytometry. **(B-D)** DCs from various genetic knockout mice were plated with β 2m cells loaded with OVA protein in the context of an ST infection for 30 minutes, followed by 2 hour treatment with gentamycin to eliminate any extracellular bacteria. CFSE labeled CD8⁺ OT-1 cells were incubated for 72 hours and assessed for proliferation (CFSE dilution) by flow cytometry. Representative data is shown as mean \pm SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, ***p<0.001, ****p<0.0001).

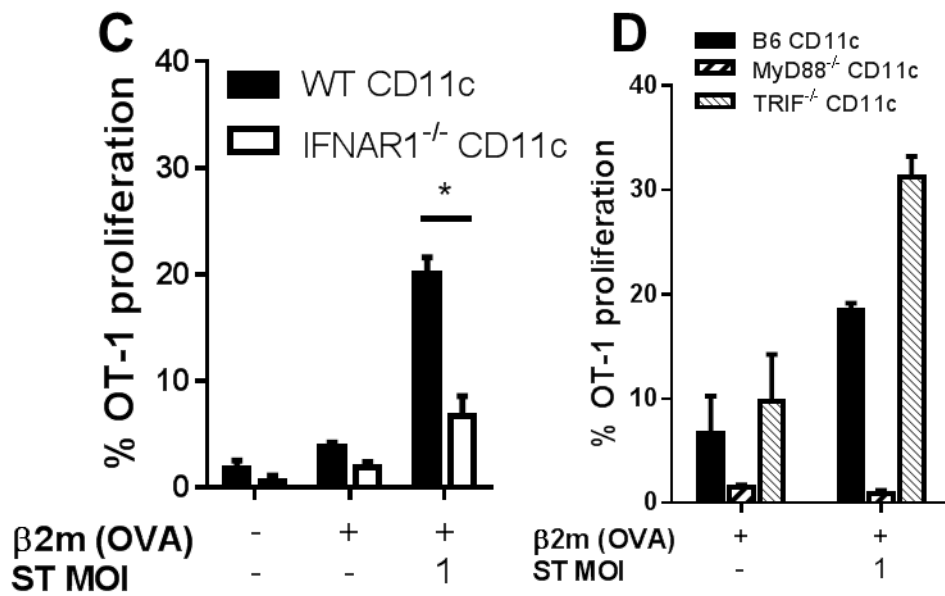
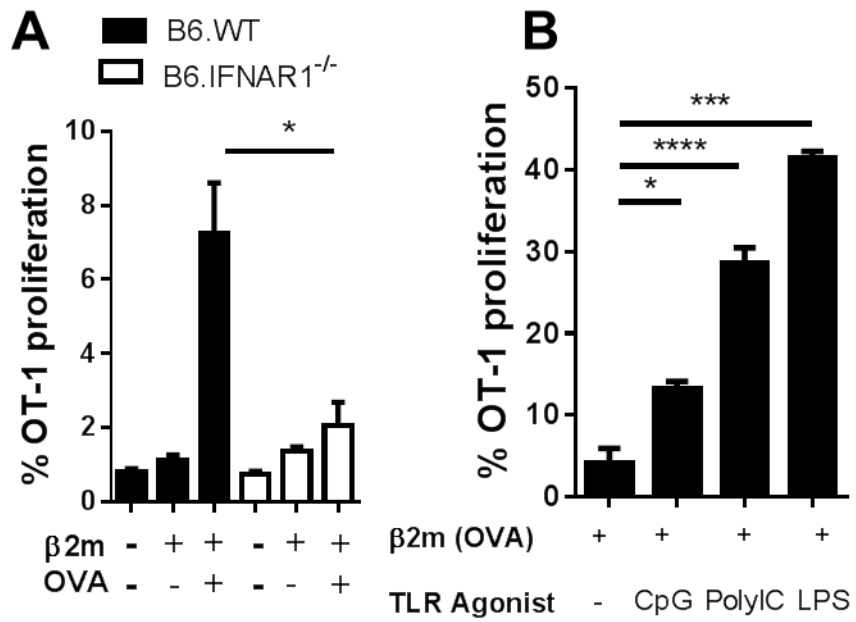
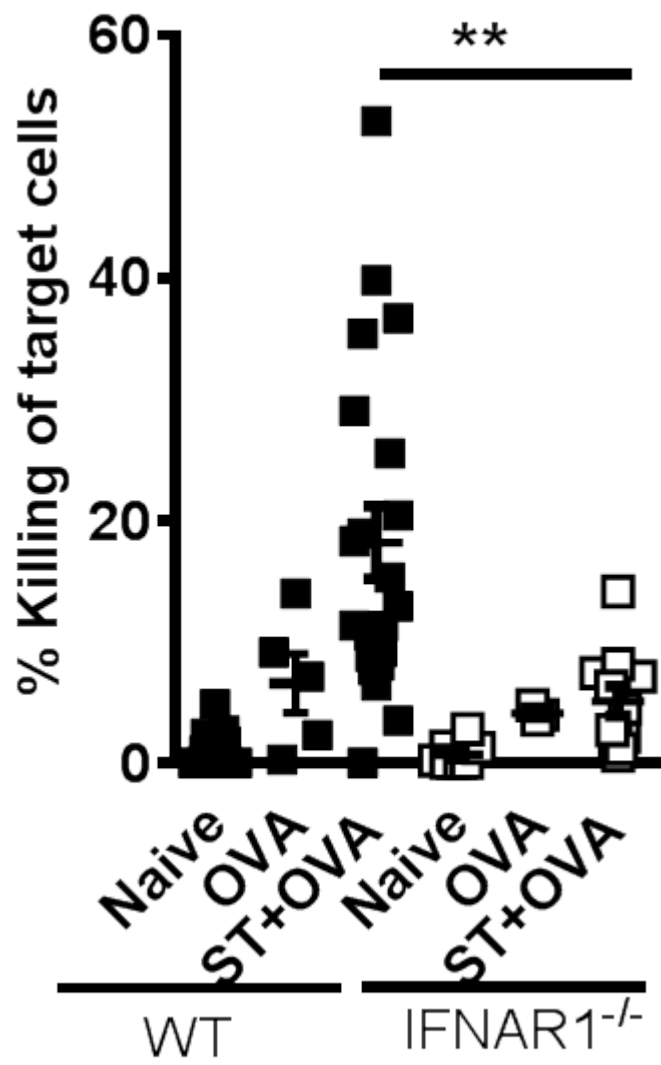


Figure 13: Priming of CD8⁺ T cells against OVA protein in the context of an ST infection requires IFN-I signalling

Wild type and IFNAR1^{-/-} mice were injected with 10³ CFU ST and 1mg of OVA protein subcutaneously at the base of the tail. On day 6 post infection, mice were injected with CFSE labeled target cells (1 million cells/i.v.). Sixteen hours after, mice were sacrificed; draining inguinal lymph nodes were collected and assessed for the presence of target cells by flow cytometry. Representative data is shown as mean ±SEM from 2-3 experiments. Statistical significance was determined by unpaired student t-test (**p<0.01).



deficient mice (**Fig. 13**). This impact requires an inflammatory stimulus, as injection of OVA alone induced poor CD8⁺ T cell response. However, ST in combination with OVA induces stronger CD8⁺ T cell response in the wild type mice and diminished response in the IFNAR1-deficient mice (**Fig 13**).

ST-OVA sequesters antigens in the phagosome of infected cells and induces rapid host fatality, similar to ST, thus not allowing sufficient time to assess the CD8⁺ T cell response. To test the impact of IFNAR signalling on CD8⁺ T cell response, I generated IFNAR1-deficient mice on 129 background and measured the endogenous CD8⁺ T cell response in the draining inguinal lymph nodes following subcutaneous ST infection. IFNAR1-deficient mice displayed decreased accumulation of cells in the lymph node at day 25 post-infection in comparison to wild type mice, indicating poor inflammatory response (**Fig. 14A**). I then assessed the priming of CD8⁺ T cells in the draining lymph nodes following subcutaneous ST infection using the ELISPOT assay but was unable to detect any difference in CD8⁺ T cell response between wild type and IFNAR1-deficient mice (**Fig. 14B**). However, enumeration of IFN- γ producing cells by ELISPOT was not sensitive, thus I utilized the *in-vivo* CTL assay. In this case, *In-vivo* killing of peptide loaded target cells indicated poor CD8⁺ T cell response in IFNAR1-deficient mice at 20 days post infection (**Fig. 14C**). Interestingly, the impact of IFNAR is only observed when ST is injected subcutaneously and the response is measured in the draining lymph node, as ST-OVA administered through the intravenous route did not show any impairment in CD8⁺ T cell response in the IFNAR1-deficient mice (**Fig. 15**).

Figure 14: IFNAR is critical for localized infection of ST

129 and 129.IFNAR1^{-/-} mice were infected with ST-OVA (10³ CFU s.c.) and assessed for CD8⁺ T cell response by *in-vivo* CTL. At day 20 post infection, **(A)** The draining lymph nodes were harvested and assessed for cell numbers. Furthermore, **(B)** IFN- γ producing cells were enumerated from the draining lymph nodes of infected mice at day 20 post infection. **(C)** Recipient mice were injected with CFSE labeled (OVA coated) target cells on day 20. Following 16 hours after administering target cells, mice were sacrificed; draining lymph nodes were harvested as assessed for the presence of CFSE labeled target cells. Representative data is shown as mean \pm SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05).

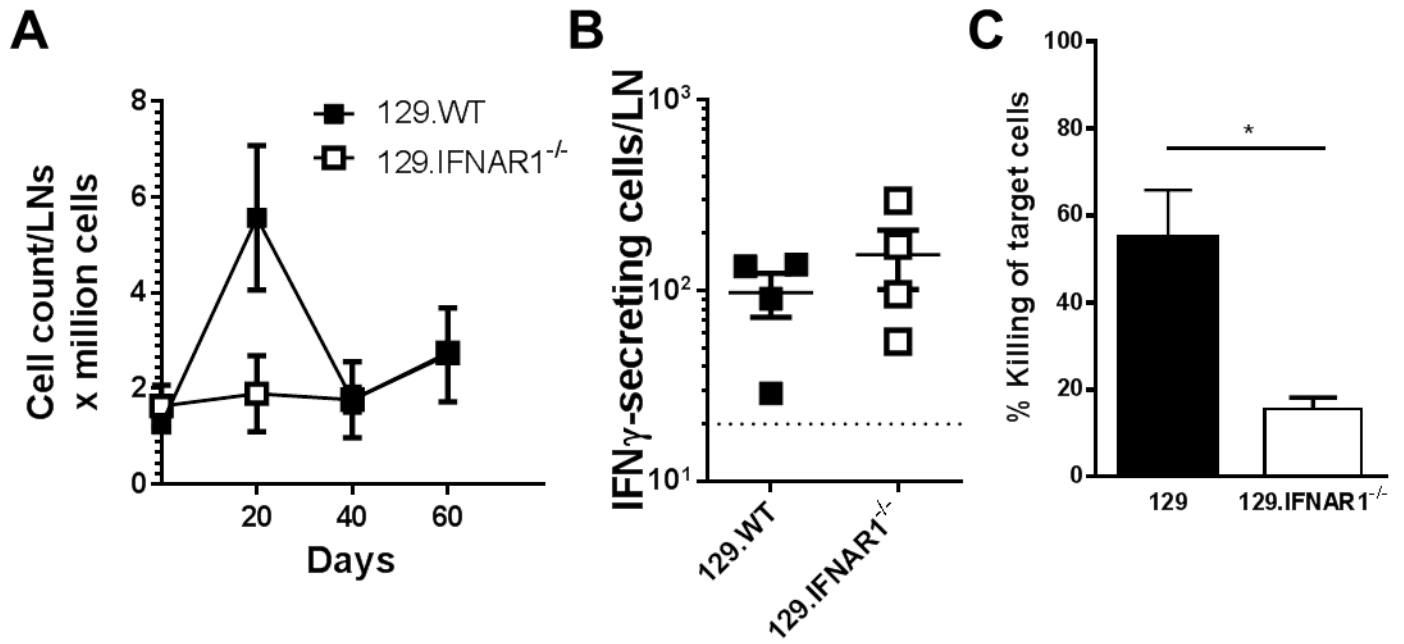
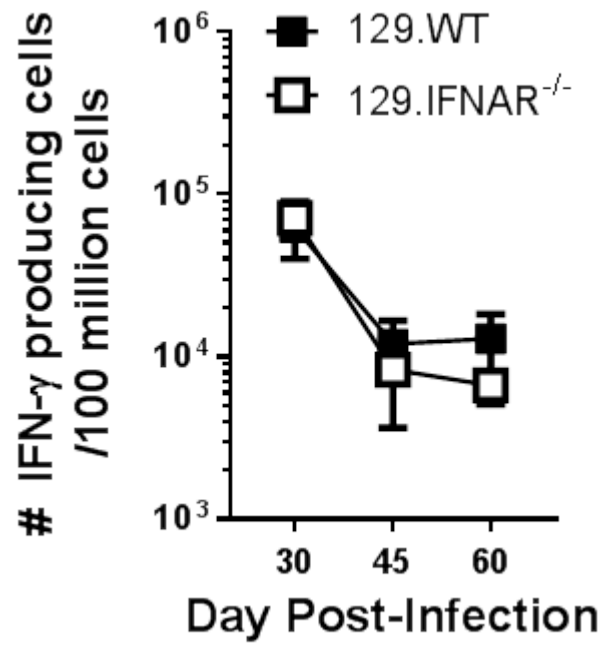


Figure 15: IFNAR signalling is not required for systemic ST infection

129 wild type and IFNAR1^{-/-} mice were infected with ST-OVA (10³ CFU *i.v.*). At various time points following infection, mice were sacrificed and spleens were harvested to assess for **(A)** IFN- γ producing cells by ELISPOT assay. All data points represent an n=3 unless otherwise stated. Representative data is shown as mean \pm SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05).

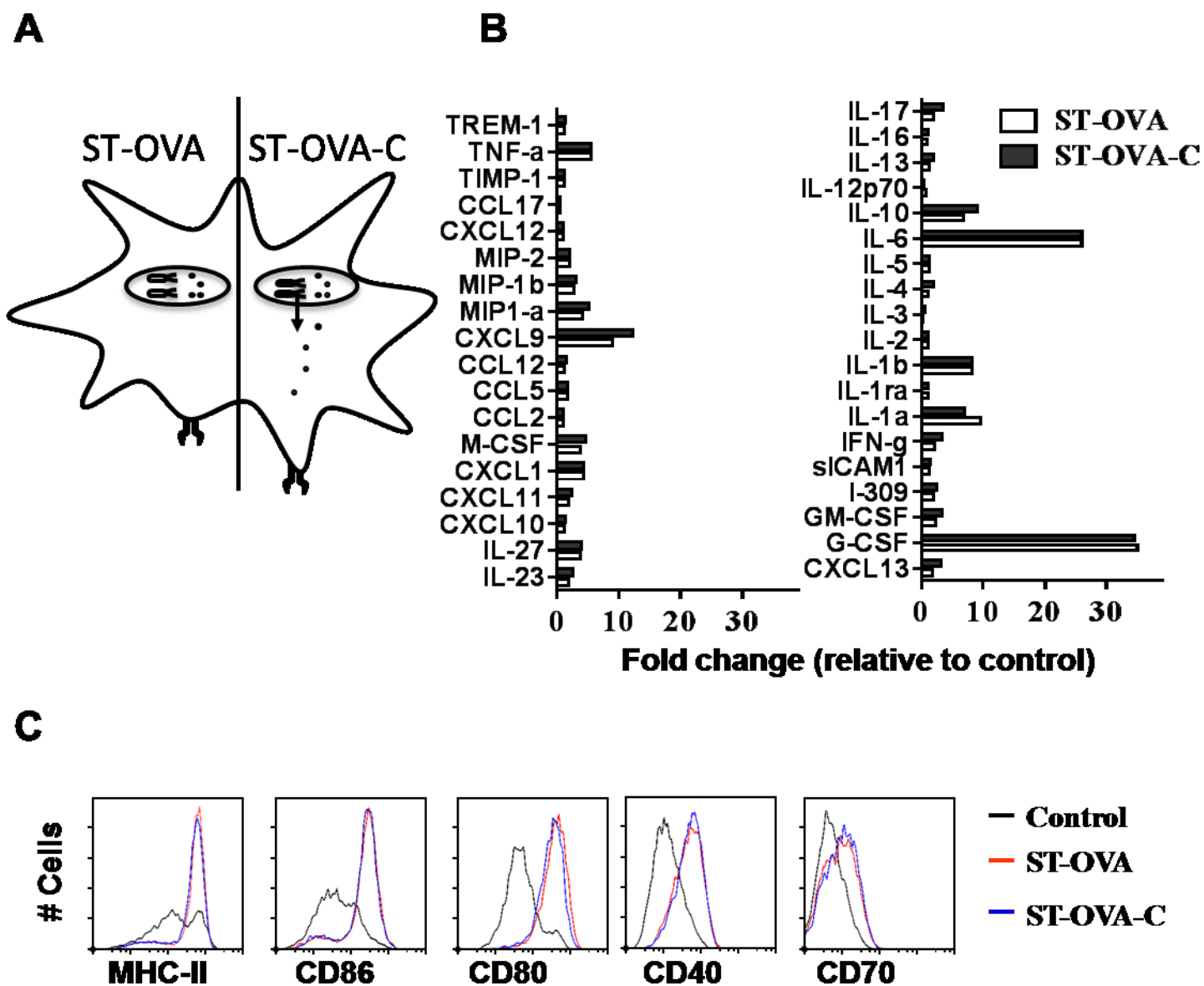


3.1.4 Critical role of IFNAR signalling in CD8⁺ T cell priming is independent of antigen location within the infected cell

ST utilizes SPI genes to create a hospitable niche in the phagosome of infected cells (166). As a consequence, all ST antigens are located in the phagosome and are sequestered from cytosolic processing of antigen. Thus the APC that acquires ST relies on cross-presentation to process the phagosomal bacteria to prime a CD8⁺ T cell response. Next I wanted to address whether the location of antigen in the context of ST infection would have an impact on the role of IFNAR in priming CD8⁺ T cells. Dr. Sad's laboratory has previously reported a recombinant of ST called ST-OVA-C, which resides in the phagosomes of infected cells but deposits recombinant OVA protein directly in the cytoplasm of infected cells through the bacterial type III secretion system (T3SS) (**Fig. 16A**). OVA deposited in the cytoplasm by ST-OVA-C engages proteasome dependent antigen processing which results in rapid priming of CD8⁺ T cells (46). Both ST-OVA and ST-OVA-C display similar infectivity on various types of immune cells (46). Following infection of DCs, both ST-OVA and ST-OVA-C induce similar cytokine and chemokine responses (**Fig. 16B**) as well as expression of MHC-II and co-stimulatory molecules (**Fig. 16C**). Therefore, I wanted to utilize the recombinant ST-OVA-C to evaluate the role of IFNAR on antigen presentation and CD8⁺ T cell priming when ST antigen engages accelerated cytosolic processing. Splenic DCs infected with ST-OVA-C engage rapid antigen presentation as measured by the proliferation of CFSE labeled OT-1 cells *in-vitro*

Figure 16: ST-OVA and ST-OVA-C are both virulent recombinant strains of ST

(A) ST-OVA maintains all antigens in the phagosome of infected cells whereas ST-OVA-C deposits antigen to the cytoplasm of infected cells. Splenic DCs were isolated from wild type mice and infected with ST, ST-OVA or ST-OVA-C for 30 minutes, followed by a 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Following 24 hours post infection, cells were assessed for expression of **(B)** production of cytokines and chemokines using a proteome assay and **(C)** MHC-II and co-stimulatory molecules by flow cytometry. Representative data is shown as mean \pm SEM from 2 experiments. Experiments outlined in panel A, B and C were performed by Dr. Fanny Tzelepis.



(**Fig. 17A, B**). In contrast, ST-OVA, which maintains OVA antigen in the phagosome (46, 161), fails to induce rapid antigen presentation and subsequent activation of OT-1 CD8⁺ T cells (**Fig 17A, B**). Both these recombinants express OVA at similar levels and both are virulent (46). Rag1-deficient mice, devoid of any T or B cell populations, succumb to infection with ST, ST-OVA or ST-OVA-C infection (10^3 CFU *i.v.*) (**Fig. 17C**).

I then addressed the impact of IFNAR signalling on CD8⁺ T cell response against ST-OVA-C. I observed a greatly reduced CD8⁺ T cell response in IFNAR1-deficient mice at day 7 post-infection (**Fig 18A**) as measured by ELISPOT assay. At day 15, the CD8⁺ T cell response between wild type and IFNAR1-deficient mice was similar in magnitude (**Fig. 18A**). The early impact of IFNAR signalling on the CD8⁺ T cell response against ST-OVA-C was further confirmed by *in-vivo* CTL killing at day 7 post-infection (**Fig. 18B**). Moreover, I observed diminished recruitment of DCs and immune cells to the draining lymph nodes of IFNAR1-deficient mice (**Fig. 18C, D**). Similar to ST-OVA, IFNAR deficiency had no impact on the CD8⁺ T cell response when ST-OVA-C was administered intravenously (**Fig. 19**). Taken together, these results indicate that IFNAR signalling promotes the development of early CD8⁺ T cell response only in a localized model, irrespective of the relative location of antigen within the APC.

3.1.5 Impact of IFNAR on CD8⁺ T cell priming is pathogen dependent

To determine if the impact of IFNAR signalling on CD8⁺ T cell priming is restricted to ST, or if this impact is observed during immune responses against

Figure 17: Recombinant strain of ST that deposits OVA into the cytoplasm of infected cells induces rapid CD8⁺ T cell response

(A) CD11c splenic DCs were plated into a 96 well plate and infected with ST-OVA or ST-OVA-C at various MOIs. CFSE labeled CD8⁺ T cells from OT-1 mice were added to control and infected DCs, and the dilution of CFSE stain was assessed 72 hours later. **(B)** Representative FACS plots are shown. **(C)** B6.Rag1^{-/-} mice were infected with ST, ST-OVA or ST-OVA-C (10² CFU i.v.) and assessed for host survival. Representative data is shown as mean ±SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (**p<0.01, ****p<0.0001). Experiments outlined in panel C were performed by Dr. Fanny Tzelepis.

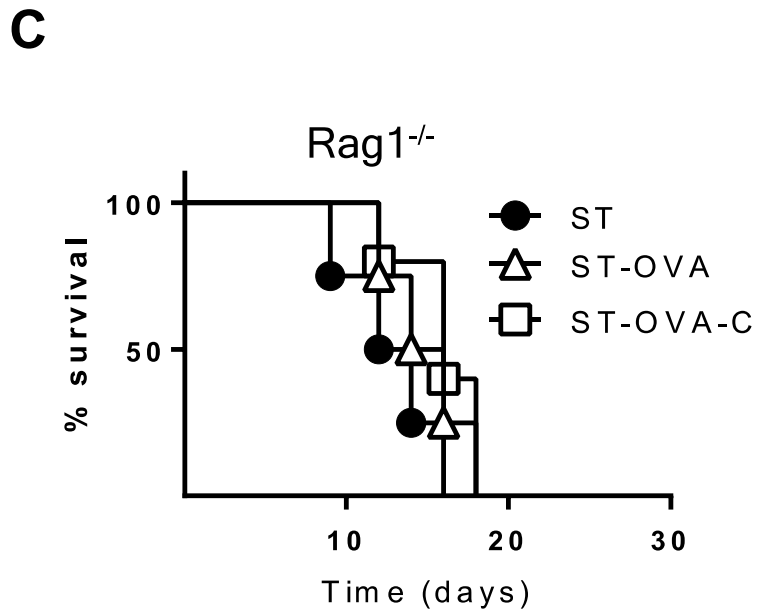
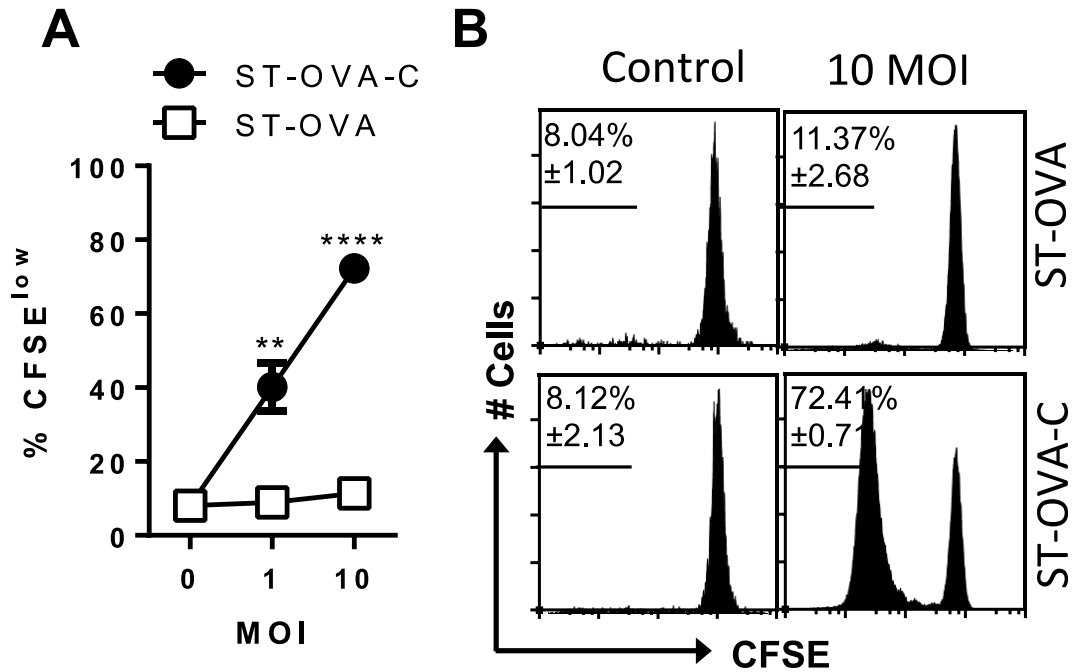


Figure 18: IFNAR1-deficient mice exhibit delayed CD8⁺ T cell kinetics against localized infection with ST-OVA-C

Wild type and IFNAR1^{-/-} mice were infected with ST-OVA-C (10³ CFU s.c.). **(A)** On day 7 and 15 following infection, mice were sacrificed; the draining lymph nodes were harvested and analyzed for IFN- γ producing cells by ELISPOT assay. **(B)** CD8⁺ T cell response was also assessed by in-vivo CTL by injecting mice infected with ST-OVA-C (10³ CFU s.c.) with CFSE labeled (SIINFEKL peptide coated) target cells on day 6. Following 16 hours after administering target cells, mice were sacrificed, spleen and draining lymph nodes were harvested as assessed for the presence of CFSE labeled target cells. **(C-D)** At various time points following infection with ST-OVA-C (10³ CFU s.c.), draining lymph nodes were harvested and assessed for the number of CD11c cells and total cell numbers. Representative data is shown as mean \pm SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05).

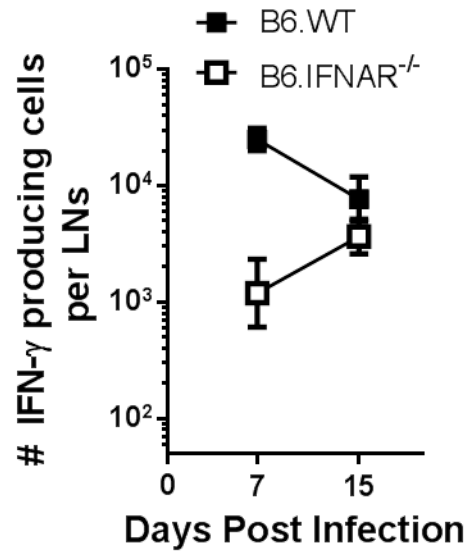
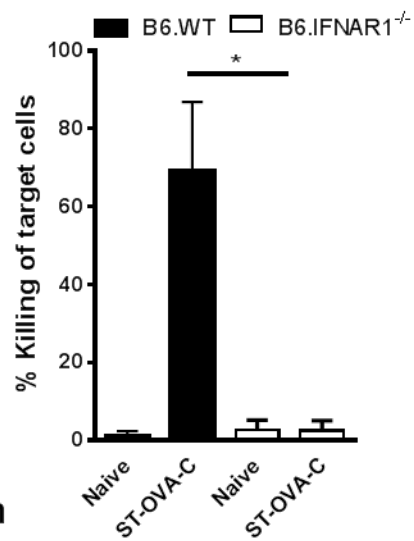
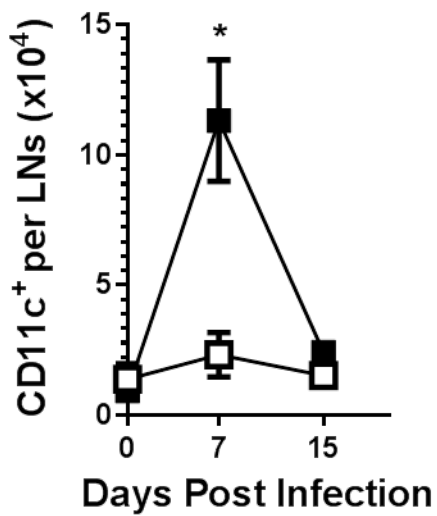
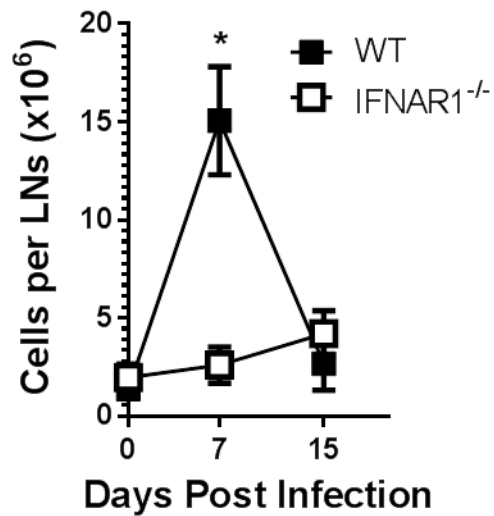
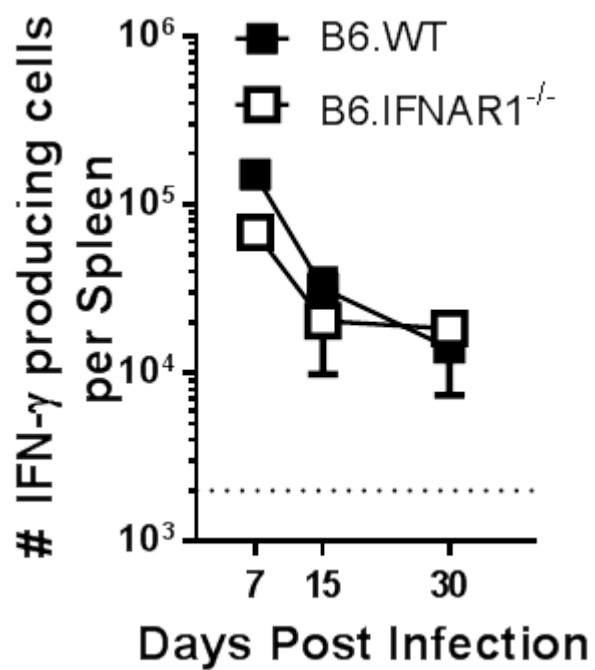
A**B****C****D**

Figure 19: IFNAR signalling has no impact on CD8⁺ T cell response against systemic ST-OVA-C

Wild type and IFNAR1^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*). At various days post infection, mice were sacrificed and the spleen was harvested and analyzed for IFN- γ producing cells by ELISPOT assay. All data points represent an n=3 unless otherwise stated. Representative data is shown as mean \pm SEM from 2-3 experiments.



other bacteria, I examined the CD8⁺ T cell response against another intracellular pathogen, LM in wild type and IFNAR1-deficient mice. LM is an intracellular bacterium which, following phagocytosis, escapes the phagosome using the virulence factor Listeriolysin (LLO) which encodes a pore-forming protein (376). LM induced a potent CD8⁺ T cell response in both wild type and IFNAR1-deficient mice as shown by ELISPOT assay (**Fig. 20A**) and *in-vivo* CTL response (**Fig. 20B**) following subcutaneous infection of LM-OVA. Furthermore, there was no noticeable influx of DCs or immune cells following LM infection in comparison to steady state levels (**Fig. 20C, D**). The CD8⁺ T cell response during systemic infection of LM-OVA was also not impacted in IFNAR1-deficient mice (**Fig. 21**). Therefore, the impact of IFNAR signalling on CD8⁺ T cell priming seems to be specific to ST infection, and is not a common phenotype observed with all intracellular bacteria.

3.1.6 Migratory DCs lacking IFNAR expression are defective in inflammatory responses and cell death

Thus far, my data suggests that IFNAR signalling may be impacting DC numbers and function independent of antigenic location. IFNAR signalling can potentially impact production of inflammatory cytokines and chemokines, which may be critical to prime a CD8⁺ T cell response and promote pathogen control of ST. To examine this, I generated BMDCs using progenitor cells from bone marrow harvested from wild type and IFNAR1-deficient mice. Bone marrow progenitors grown in the presence of GM-CSF for 8 days give rise to GM-CSF-DCs, which closely resemble monocyte derived DCs *in-vivo* (**Fig. 8**) (360). Bone **Figure 20:**

Figure 20: IFNAR signalling is not required for CD8⁺ T cell responses against localized LM infection

Wild type and IFNAR1^{-/-} mice were infected with LM-OVA (10⁴ CFU s.c.). **(A)** On day 7 and 15 following infection, mice were sacrificed; the draining lymph nodes were harvested and analyzed for IFN- γ producing cells by ELISPOT assay. **(B)** CD8⁺ T cell response was also assessed by in-vivo CTL by injected mice infected with LM-OVA (10³ CFU s.c.) with CFSE labeled (OVA₂₅₇₋₂₆₄ coated) target cells on day 6. Following 16 hours after administering target cells, mice were sacrificed; draining lymph nodes were harvested as assessed for the presence of CFSE labeled target cells. **(C-D)** At various time points following infection with ST-OVA-C (10³ CFU s.c.), draining lymph nodes were harvested as assessed for the number of CD11c cells and total cell numbers. All data points represent an n=3 unless otherwise stated. Representative data is shown as mean \pm SEM from 2 experiments.

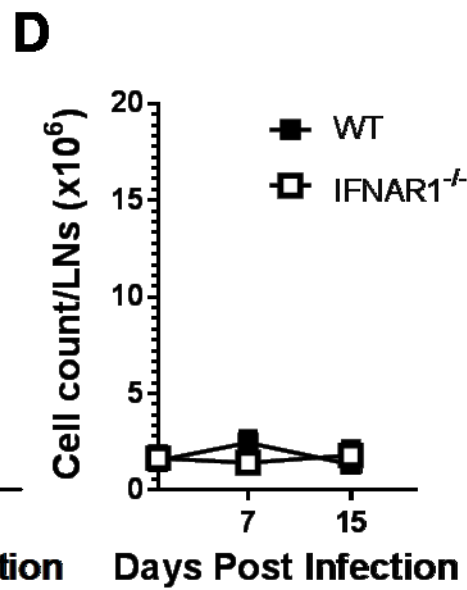
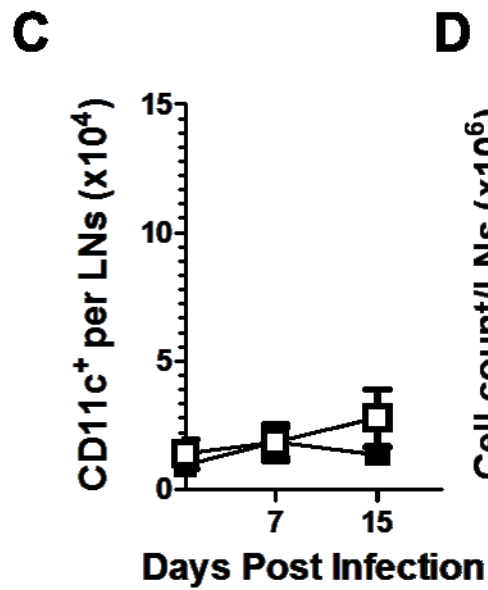
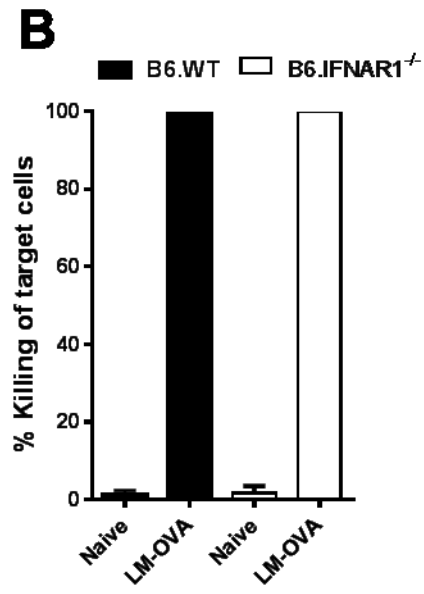
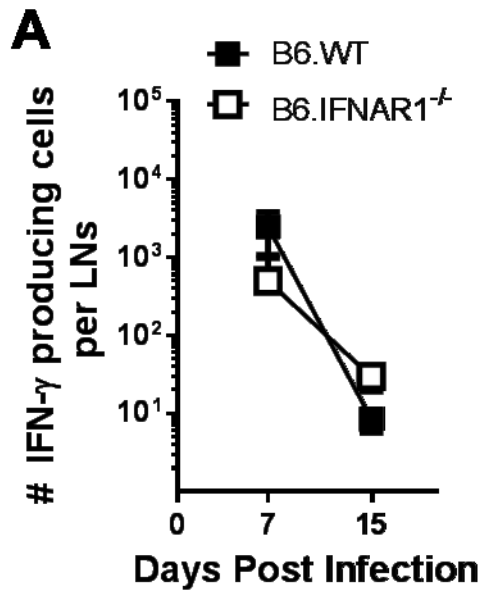
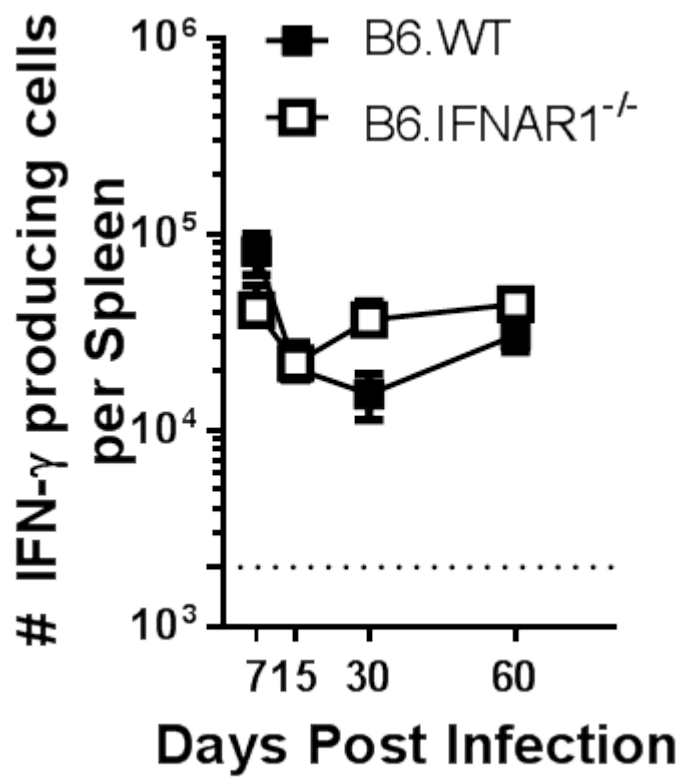


Figure 21: IFNAR has no impact on CD8⁺ T cell response against systemic LM

Wild type and IFNAR1^{-/-} mice were infected with LM-OVA (10⁴ CFU *i.v.*). At various days post infection, mice were sacrificed and the spleen was harvested and analyzed for IFN- γ producing cells by ELISPOT assay. All data points represent an n=3 unless otherwise stated. Representative data is shown as mean \pm SEM from 2 experiments.



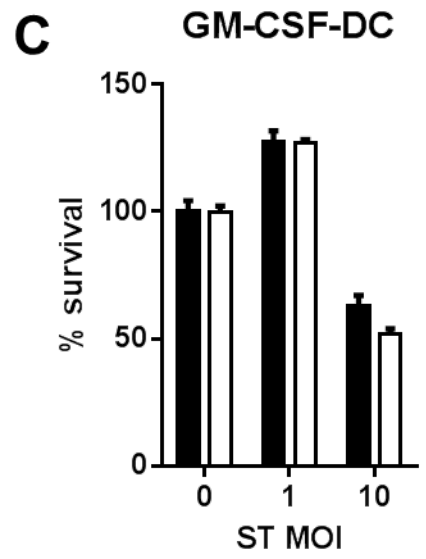
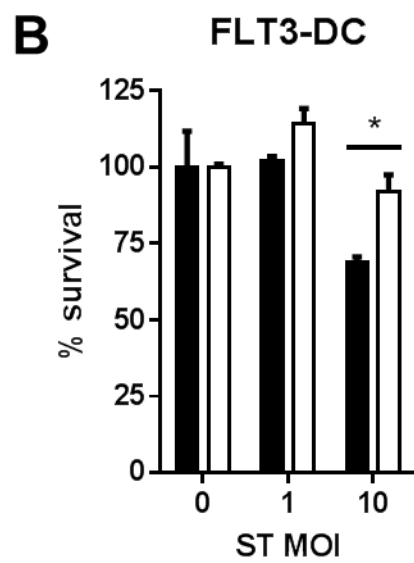
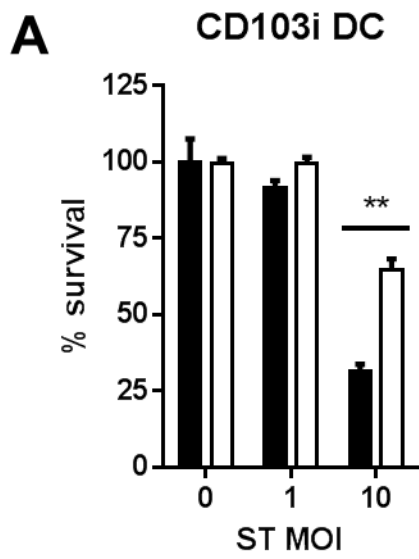
marrow progenitors grown in the presence of the growth factors FLT3L and GM-CSF for 8 days *in-vitro*, promote the growth of a migratory subset of DCs called CD103i DC (**Fig. 8**) (363). Lastly, bone marrow progenitors grown in the presence of FLT3L for 10 days promotes the growth of an *in-vitro* culture of DCs (called FLT3L-DCs) that closely resembles CD8 α DC, which specializes in cross-presentation (**Fig. 8**) (362). However, bone marrow FLT3L cultures also promote the growth of pDCs and CD11b DCs (**Fig. 8**) (362).

To determine the specific impact of IFNAR signalling on different subsets of DCs, I infected each *in-vitro* subset of DCs with ST and examined the importance of IFNAR on cell survival and its impact on the production of cytokines and chemokines. I infected wild type and IFNAR1-deficient CD103i DCs and measured cell death at 24 h post infection using the neutral red assay, and noted substantial resistance of IFNAR1-deficient CD103i DCs to cell death following ST infection (**Fig. 22A**). A similar impact was observed with FLT3L-DCs (**Fig. 22B**). Interestingly, both wild type and IFNAR1-deficient GMCSF-DCs were highly susceptible to cell death upon ST infection (**Fig. 22C**).

I measured a panel of inflammatory cytokines and chemokines from the supernatants of infected DCs collected at 24 hours. I observed that IFNAR1-deficient CD103i DCs had a diminished ability to produce inflammatory cytokines such as IL-1 β , TNF and IL-6 at multiplicity of infection (MOI) where cell death was not different between wild type and IFNAR1-deficient CD103i DCs (**Fig. 23A**). Similarly, inflammatory chemokines such as MIP-1 α , MIP-1 β and MIG were also diminished in IFNAR1-deficient CD103i DCs as compared to wild type CD103i

Figure 22: CD103i DCs require IFNAR to induce ST mediated cell death

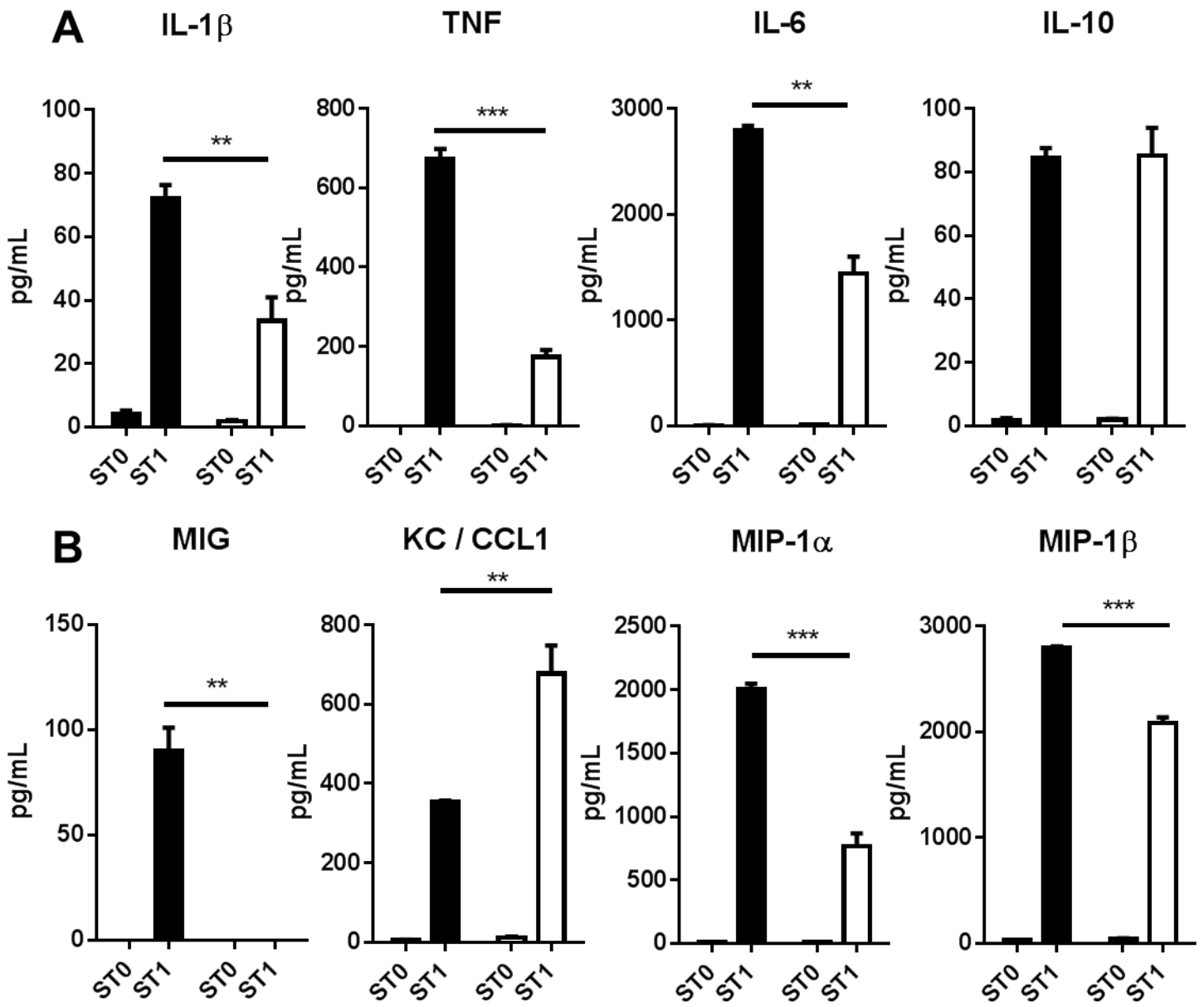
(A-C) Wild type and IFNAR1^{-/-} CD103i DCs, FLT3L-DCs and GM-CSF-DCs were infected with ST at various MOIs for 30 minutes, followed by 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Cells were incubated for 24 hours and then assessed by cell viability by neutral red assay. Representative data is shown as mean \pm SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01).



■ WT □ IFNAR1^{-/-}

Figure 23: CD103i DCs require IFNAR signalling for optimal cytokine and chemokine production following ST infection

Wild type and IFNAR1^{-/-} CD103i DCs were infected with ST at various MOIs for 30 minutes, followed by 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Cells were incubated for 24 hours, supernatants were collected and assessed for **(A)** cytokine and **(B)** chemokine production. Representative data is shown as mean \pm SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001).



DCs (**Fig. 23B**). However, IFNAR deficiency had no impact on the production of RANTES and IL-10 following ST infection (**Fig 23A, B**). KC was significantly higher in IFNAR1-deficient CD103i DCs following ST infection, which agrees with reports that suggest KC is inhibited by IFN α R1 signalling (**Fig. 23B**) (377). Furthermore, I examined the impact of IFNAR signalling on cytokine and chemokine production in wild type and IFNAR1-deficient GMCSF-DCs following ST infection. IFNAR1-deficient GMCSF-DCs expressed diminished levels of cytokines IL-10, IL-6, IL-1 β , TNF- α and chemokines such as MIP-1 α and MIP-1 β following ST infection (**Fig. 24A-B**).

To evaluate the impact of IFNAR signalling on DC migration, I assessed DC recruitment to the draining lymph nodes following ST infection at various time intervals post infection. Interestingly, there was a minimal impact during the early stages of infection. I observed high influx of DCs and other immune cells in the draining lymph nodes of wild type mice at day 7, whereas the IFNAR1-deficient mice exhibited minimal influx of cells in the draining lymph nodes (**Fig. 25A-C**). This may be a consequence of IFNAR1-deficient cells expressing reduced levels of inflammatory cytokines (**Fig. 23, 24**), which results in poor recruitment of immune cells to the draining lymph nodes (**Fig. 18, 25**) that may diminish an optimal CD8⁺ T cell response.

Overall, my data indicates that IFNAR signalling is critical to mount a CD8⁺ T cell response against localized ST infection. IFNAR signalling in DCs was required for cytokine and chemokine as well as cell death following ST infection. Interestingly, this impacted the recruitment of migratory and resident DCs to the

Figure 24: GMCSF-DCs require IFNAR signalling for cytokine and chemokine production following ST infection

Wild type and IFNAR1^{-/-} GMCSF-DCs were infected with ST at various MOIs for 30 minutes, followed by 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Cells were incubated for 24 hours, supernatants were collected and assessed for **(A)** cytokine and **(B)** chemokine production. Representative data is shown as mean ±SEM from 2 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001).

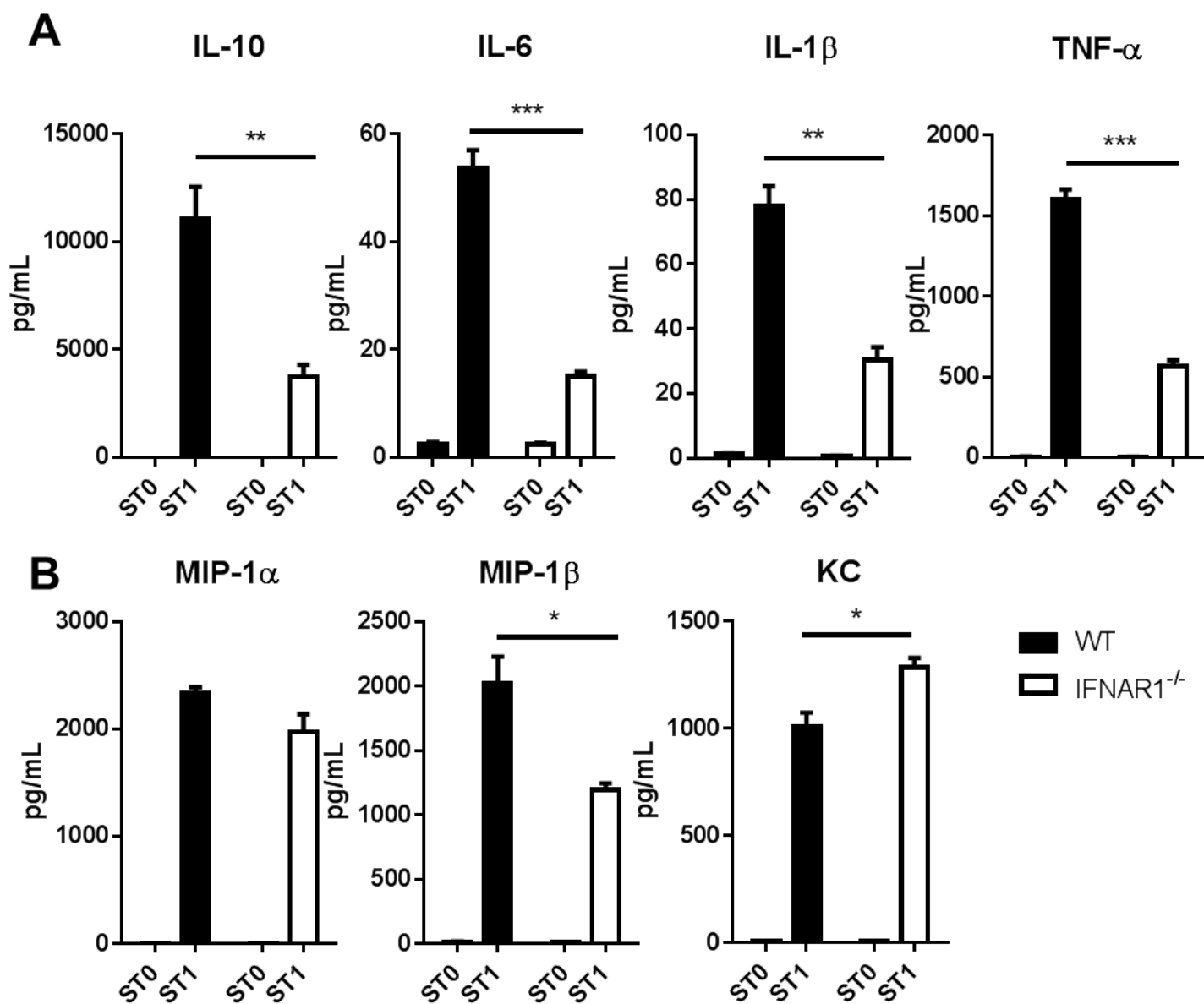
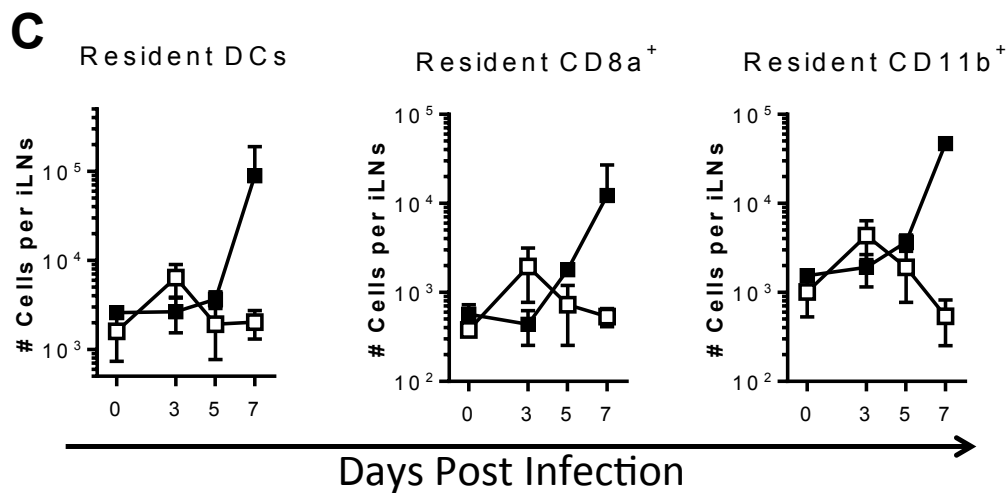
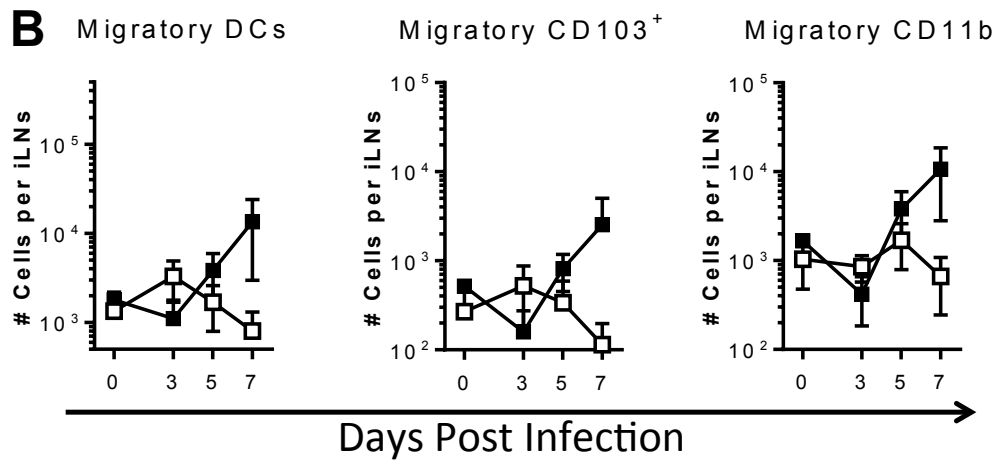
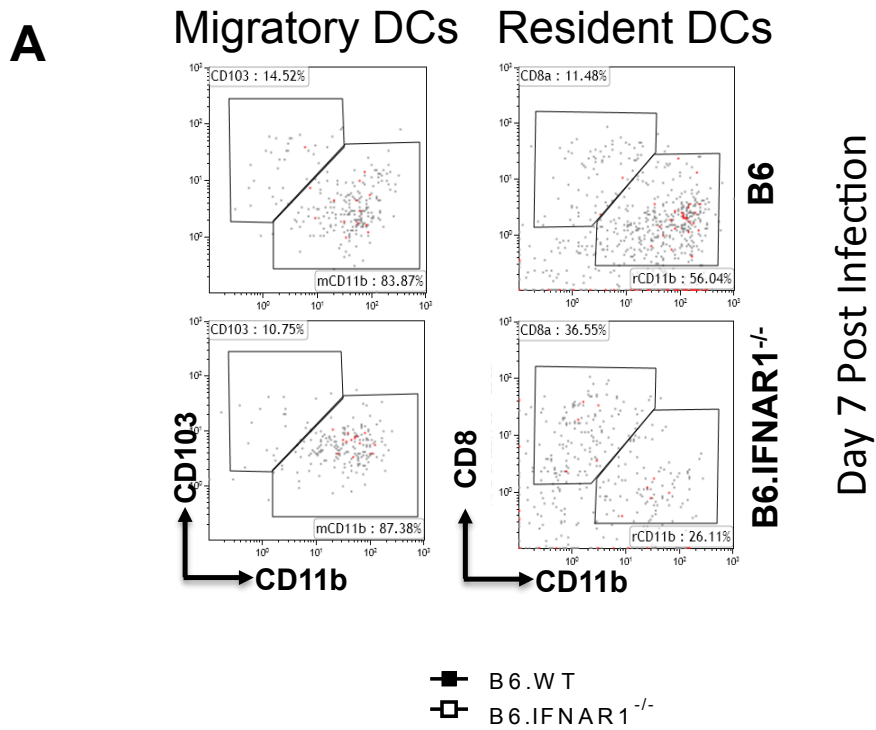


Figure 25: Reduced infiltration of migratory and resident DCs in the draining lymph nodes of IFNAR1-deficient mice following ST-OVA-C infection

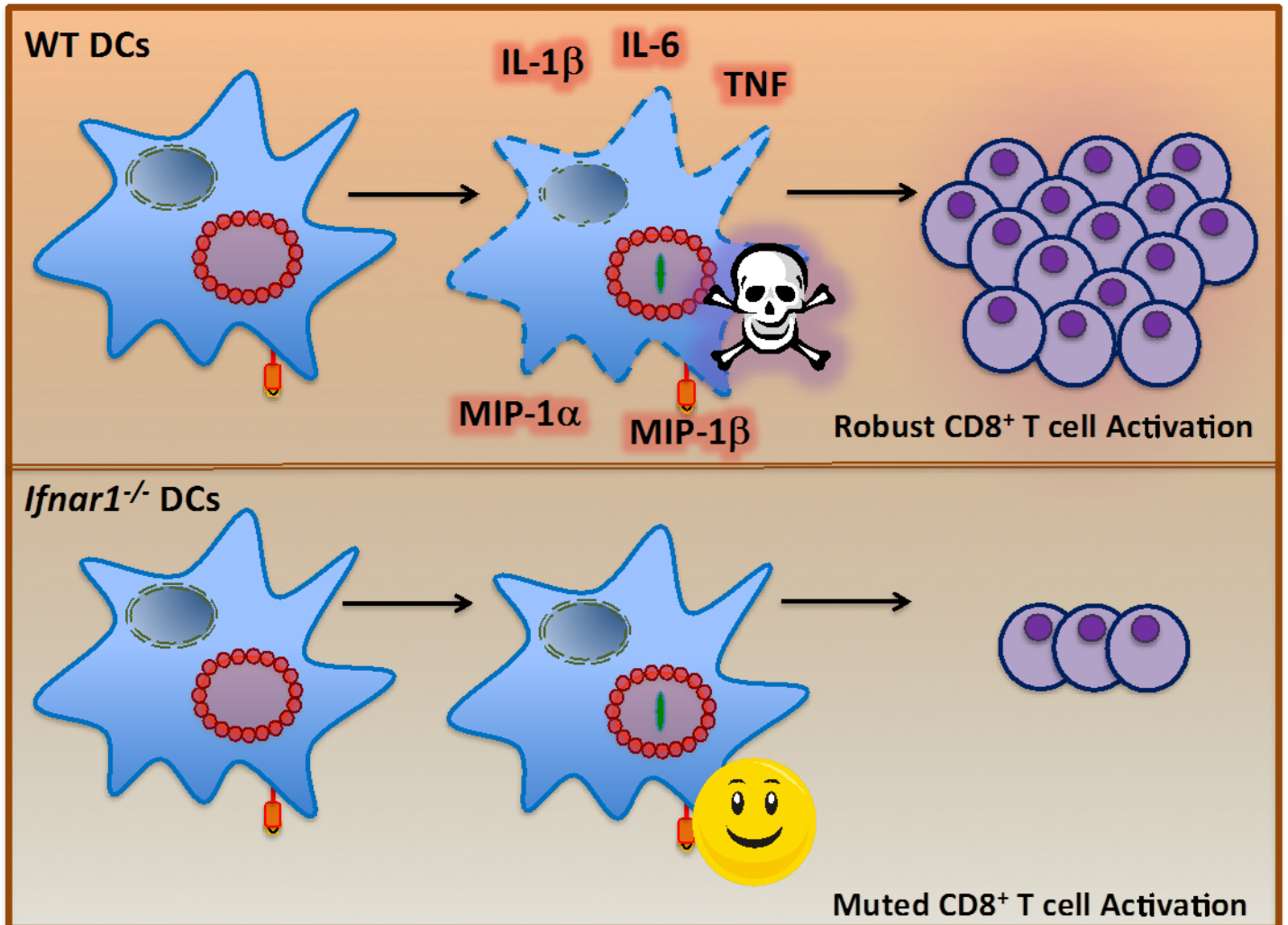
Wild type and IFNAR1^{-/-} mice were infected with ST-OVA-C (10³ CFU s.c.). **(A)** At various days post infection, mice were sacrificed, the draining lymph nodes was harvested and analyzed for different DC populations by flow cytometry **(B-C)** Total number of migratory and resident DC subset populations were assessed by flow cytometry and total cell numbers. All data points represent an n=3 unless otherwise stated. Representative data is shown as mean ±SEM from 2 experiments.



draining lymph nodes of infected mice. This impact was restricted to ST, as infection with another intracellular bacterium, LM, was not attenuated in IFNAR1-deficient mice. However, when the response was measured in a systemic infection model, the CD8⁺ T cell response was not dependent on IFNAR signalling, even in the ST infection model. Overall, these results indicate that IFNAR signalling was required to mount an early CD8⁺ T cell response against ST in the draining lymph nodes (**Fig. 26**).

Figure 26: Visual Abstract I: IFNAR signalling is required for protective CD8⁺ T cell response against ST

IFNAR signalling is critical for innate and adaptive immune responses against a variety of pathogens. Our data suggests that IFNAR signalling is critical to mount a CD8 T cell response against recombinants of ST in the draining lymph nodes of infected mice. IFNAR signalling is critical for inflammatory cytokine and chemokine production following ST infection. Furthermore, IFNAR signalling mediates the cell death in migratory CD103⁺ DC following ST infection, which may be a critical source of cellular antigen in the draining lymph nodes to promote antigen-presentation and promote a robust CD8⁺ T cell response. Collectively, our data suggests that IFNAR signalling is critical for immune protection against ST by mediating inflammation, cell death and antigen-presentation to mount a protective CD8⁺ T cell response.



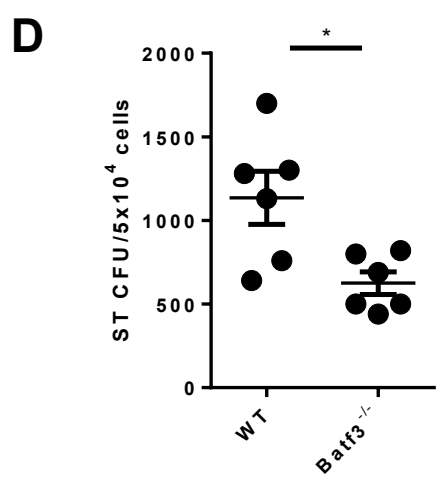
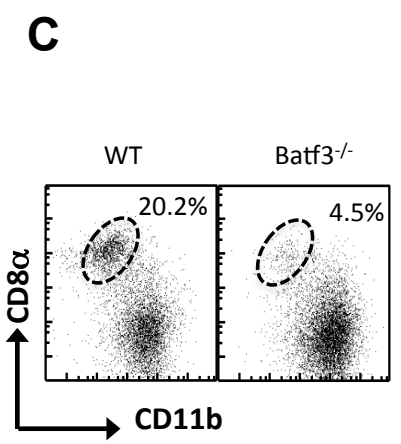
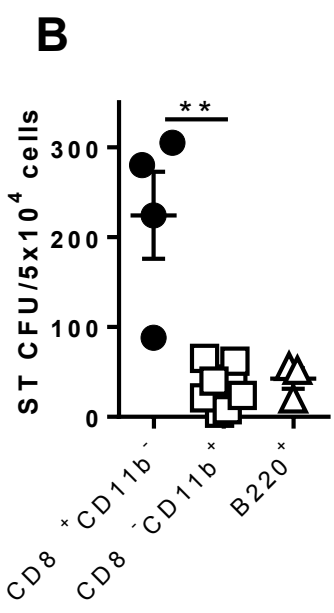
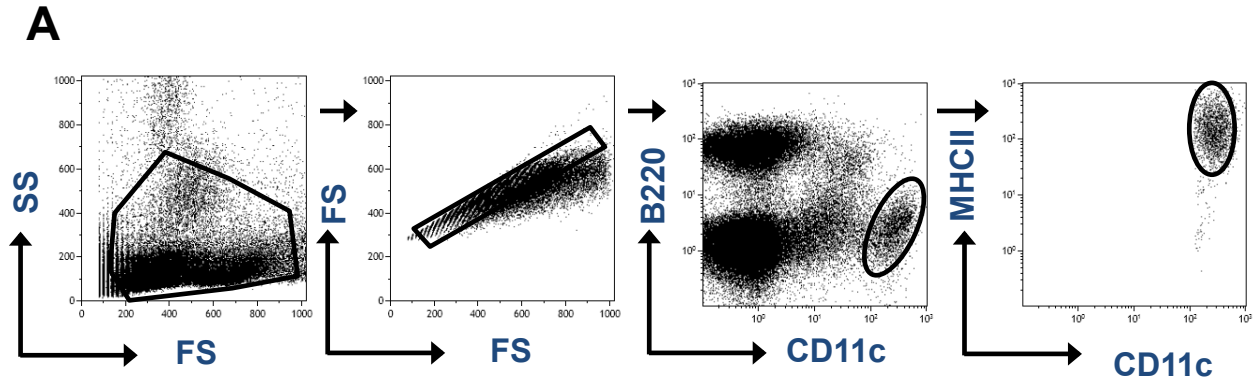
3.2 CHAPTER 2: Batf3-dependent DCs are critical for development of CD8⁺ T cell responses against *Salmonella* Typhimurium regardless of the location of antigen

3.2.1 CD8 α DCs display increased uptake of ST antigen

Since ST derived antigens are sequestered into the phagosome of infected APCs, CD8⁺ T cell response against phagosomal pathogens are most likely primed through the cross-presentation pathway, which allows for MHC class-I presentation of phagosomal antigen (378). CD8 α DCs have been shown to be the most important population of DCs that promotes cross-presentation (52, 101, 125, 379). Therefore, I evaluated the role of CD8 α DCs against the immune response against ST. Following infection of sorted CD8 α DC, CD11b DC and pDC populations *in-vitro*, I observed increased uptake of ST by CD8 α DCs in comparison to other DC subsets at 2 hours post-infection (**Fig. 27A-B**). Due to the limited incubation time of 2 hours, the increased bacterial burden in CD8 α DCs can be attributed to enhanced uptake of ST in contrast to enhanced bacterial proliferation within CD8 α DC populations (the intracellular doubling time of ST is 7 hours). I also utilized the B6.Batf3-deficient mouse model, which lacks Batf3, a key AP-1 transcription factor required for development of CD8 α DCs during steady state (101). Furthermore, I crossed the B6.Batf3-deficient mice with B6.Nramp mice to create the B6.Nramp.Batf3-deficient mouse strain. This would allow me to assess the impact of Batf3-dependent DCs on the CD8⁺ T cell

Figure 27: ST is selectively acquired by Batf3-dependent DCs

(A) Splenic DCs were quantified by flow cytometry using cell surface staining. DCs were characterized by gating onto singlet cells based on forward and side scatter. DCs were gated as CD11c⁺B220⁻MHC-II^{hi} cells. Splenic DCs were quantified as CD8 α DCs and CD11b DCs. **(B)** Splenic DCs were sorted into CD8 α DCs, CD11b DCs and pDC (CD11c⁺B220^{lo/+}). Each DC subset was infected with ST at an MOI of 10 for 30 min, followed by gentamycin treatment for 2 hours. Cells were lysed using lysis buffer and plated onto BHI plates to assess the bacterial counts in each DC subset. **(C)** Batf3^{-/-} mice backcrossed onto the Nramp background were virtually devoid of any CD8a DCs. Representative FACS plots of CD8 α and CD11b DC populations from B6.Nramp and B6.Nramp.Batf3^{-/-} mice are shown. **(D)** CD11c DCs isolated from wild type and Batf3^{-/-} spleens were plated into 96 well plates and infected with 10 MOI of ST for 30 min followed by gentamycin treatment. Bacterial counts in the cells were then evaluated following 2 hours post infection following treatment with lysis buffer. Representative data is shown as mean \pm SEM from 3 experiments. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01).



response against ST as the mice will not rapidly succumb to infection. As expected, B6.Nramp.Batf3-deficient mice lack CD8 α DC in the spleen (**Fig 27C**). To determine the ability of different DC populations to acquire ST antigens, I purified splenic wild type and Batf3-deficient DCs and infected them with ST *in-vitro*. As expected, wild type DCs acquired more ST bacteria than Batf3-deficient DCs following 2 hours of infection (**Fig. 27D**).

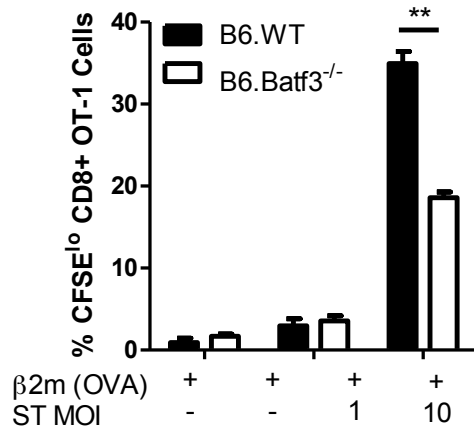
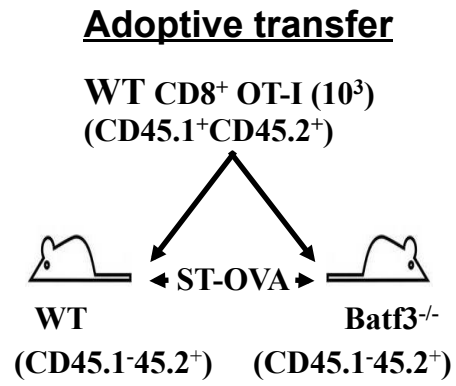
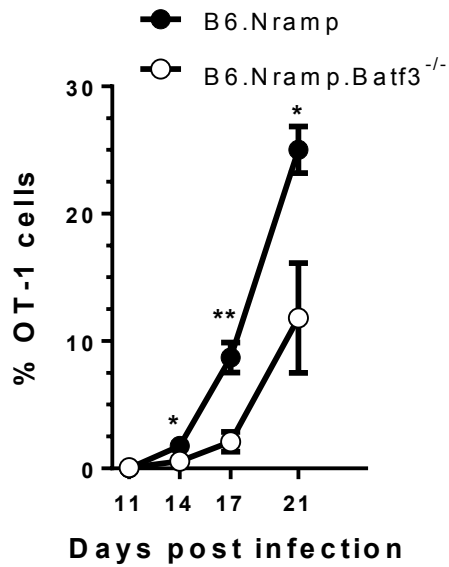
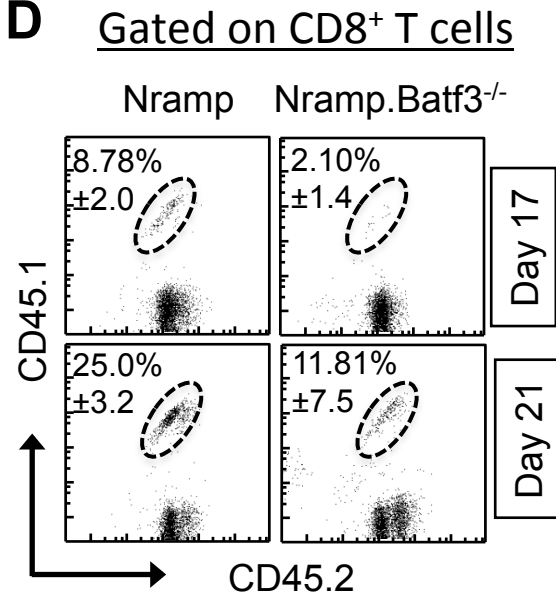
3.2.2 Poor CD8⁺ T cell response in Batf3-deficient mice

Pathogens such as LM can induce a strong CD8⁺ T cell response towards highly immunogenic epitopes. For instance, the pore forming toxin Listeriolysin O (LLO) is a highly immunogenic antigen which induces a strong CD4⁺ and CD8⁺ T cell response following infection with LM (380). In stark contrast, ST induces a delayed CD8⁺ T cell response *in-vivo* (161), possibly due to the sequestration of ST antigens in the phagosome. Consequently, there are no reports of a highly immunogenic CD8⁺ T cell epitope derived from ST, which can be used to track a cytotoxic T cell response. Thus, I utilized recombinants of ST that express the highly immunogenic model antigen OVA to evaluate the CD8⁺ T cell response against ST.

To determine the impact of the transcription factor Batf3 on antigen presentation against ST DCs in mounting a CD8⁺ T cell response against ST, I first assessed the capacity of wild type and Batf3-deficient DCs to cross-present OVA antigen *in-vitro* (**Fig 28A**). As expected, Batf3-deficient DCs had reduced capacity to cross-present cellular antigen in the context of an ST infection. To

Figure 28: Delayed CD8⁺ T cell kinetics against ST infection in Batf3 deficient mice

(A) B6.Nramp and B6.Nramp.Batf3^{-/-} DCs were plated into 96 well plates at a concentration of 10⁵ cells per well. DCs were co-incubated with β2m^{-/-} cells loaded with OVA protein and ST at various MOIs for 30 minutes, followed by a 2 hour gentamycin wash. CFSE labelled OT-1 CD8⁺ T cells were added and proliferation of OT-1 cells was evaluated following 72h of infection by flow cytometry as a measure of cross-presentation of cellular antigen **(B)** CD8⁺ OT-1 cells (CD45.1⁺CD45.2⁺) were injected (10⁵ *i.v.*) into B6.Nramp and B6.Nramp.Batf3-deficient mice (endogenous cells expressing CD45.1⁻CD45.2⁺). Two days following infection, mice were infected with ST-OVA (10³ *i.v.*). **(C)** Blood was collected from recipient mice through saphenous bleeding at various time points and assessed for the numbers of OT-1 (CD8⁺CD45.1⁺CD45.2⁺) CD8⁺ T cells using flow cytometry and cell surface staining. **(D)** Representative FACS plots are shown. Representative data is shown as mean ±SEM from 2-4 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student-t test (*p<0.05, **p<0.01).

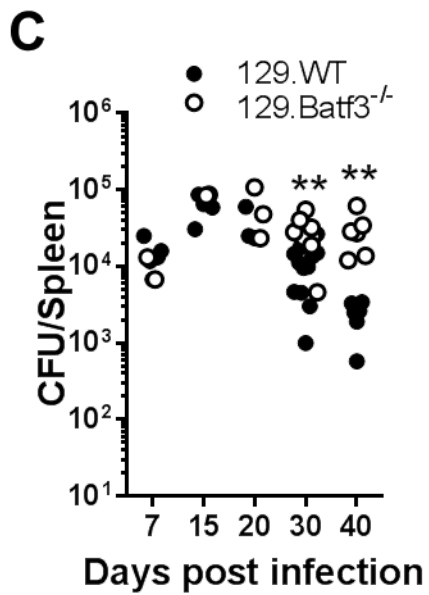
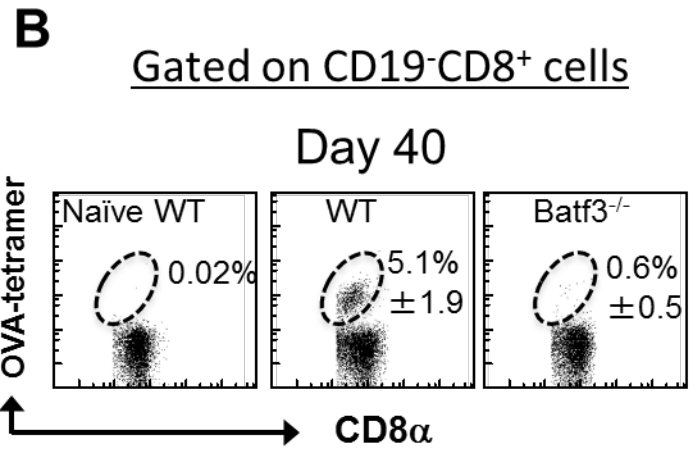
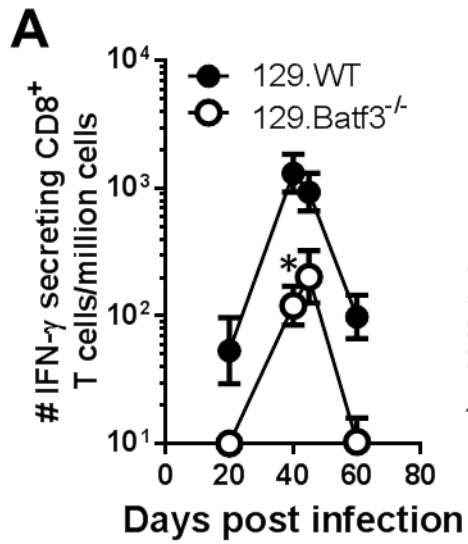
A**B****C****D**

further assess the impact of Batf3 on CD8⁺ T cell responses against ST, I adoptively transferred purified OT-1 transgenic CD8⁺ T cells (with a transgenic TCR that can bind H-2Kb-OVA₂₅₇₋₂₆₄) onto Nrap and Nrap.Batf3-deficient mice (**Fig. 28B**). Recipient wild type and Batf3-deficient mice express CD45.1⁺ CD45.2⁺ while the OT-1 CD8⁺ T cells express CD45.1⁺CD45.2⁺. Following infection with ST-OVA, I monitored the expansion of endogenous CD8⁺ T cells or adoptively transferred OT-1 CD8⁺ T cells using differential expression of CD45 (**Fig. 28C**). Wild-type mice generated a delayed, but robust CD8⁺ T cell response within 21 days post infection. In contrast, the response in Batf3-deficient mice was significantly attenuated (**Fig. 28C,D**). The CD8⁺ T cell response in Batf3-deficient mice was significantly reduced at all the time points tested. By monitoring OT-1 CD8⁺ T cell proliferation in wild type and Batf3-deficient hosts, these results suggest that the impact of Batf3 in CD8⁺ T cell priming is not cell-intrinsic to CD8⁺ T cells and is most likely an impact within the APCs.

To determine if Batf3 is also required to prime CD8⁺ T cells during a chronic ST infection in resistant mice, I infected 129 and 129.Batf3-deficient mice with ST-OVA (10³ CFU *i.v.*) and evaluated the CD8⁺ T cell response at various time points post infection by ELISPOT assay. As expected, CD8⁺ T cell response was reduced in Batf3-deficient mice throughout the duration of the infection (**Fig. 29A**). Evaluation of CD8⁺ T cell response by H-2K^b-OVA₂₅₇₋₂₆₄ tetramer staining further indicated that Batf3 promoted the development of a potent CD8⁺ T cell response against ST-OVA (**Fig. 29B**). Furthermore, 129.Batf3-deficient mice had

Figure 29: Batf3 promotes CD8⁺ T cell response against chronic ST infection

129.WT and 129.Batf3^{-/-} mice were infected with ST-OVA (10³ *i.v.*) and monitored for 60 days. **(A)** Spleens were harvested and assessed for the numbers of OVA₂₅₇₋₂₆₄-specific, IFN-γ producing cells at various time points by ELISPOT assay. **(B)** At day 40, the peak of response, we also evaluated the numbers of H-2K^b-OVA₂₅₇₋₂₆₄ tetramer positive cells within the CD8⁺ T cell gate by flow cytometry. **(C)** Bacterial burden in the spleens of 129.WT and 129.Batf3^{-/-} mice was also assessed at various time points. Representative data is shown as mean ±SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student-t test (**p<0.05, ***p<0.01).



higher bacterial burden at time periods following the peak of CD8⁺ T cell response in comparison to wild type mice (**Fig. 29C**).

Next I wanted to address whether antigenic location would have an impact on the role of the transcription factor Batf3 in priming CD8⁺ T cells. Thus, I addressed the impact of Batf3 on antigen presentation against ST-OVA-C. Interestingly, both wild type and Batf3-deficient DCs were able to induce potent antigen presentation against ST-OVA-C *in vitro* (**Fig. 30A**), suggesting that lack of the subset of CD8 α DCs does not impact direct presentation of cytosolic OVA *in-vitro*. Surprisingly, infection of Batf3-deficient B6.Nramp mice with ST-OVA-C (10³ CFU *i.v.*) resulted in delayed CD8⁺ T cell response during the early stage of infection (**Fig. 30B-C**). At later time points (15 days post infection), the CD8⁺ T cell response between wild type and Batf3-deficient mice was similar. This effect of Batf3 on cytotoxic CD8⁺ T cell activation was not cell intrinsic to CD8⁺ T cells as OT-1 CD8⁺ T cells transferred to Batf3-deficient hosts displayed poor proliferation during the initial phase of ST-OVA-C infection (**Fig. 31A-C**). An interesting result that emerges from my studies in Batf3-deficient mice is that during infection with ST-OVA, where antigen is confined to the phagosomal location, CD8⁺ T cell response is blunted throughout the duration of infection in Batf3-deficient mice, whereas in case of the cytosolic antigen, ST-OVA-C, Batf3 is critical only during the initial phase of infection.

Figure 30: Batf3 is important for early CD8⁺ T cell priming against ST when antigen is translocated to the cytoplasm

(A) B6.Nramp and B6.Nramp.Batf3^{-/-} DCs were plated onto 96 well plates at a concentration of 10⁵ cells per well and infected with ST-OVA-C at various MOIs for 30 minutes, followed by a 2 hour gentamycin wash. CFSE labeled OT-1 CD8⁺ T cells were added and evaluated at 72h post infection by flow cytometry. **(B)** B6.Nramp and B6.Nramp.Batf3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*) and assessed for the numbers of OVA₂₅₇₋₂₆₄-specific, IFN-γ-secreting cells at various time points by ELISPOT assay using splenic cells. **(C)** Representative ELISPOT wells used to quantify IFN-γ producing cells are shown. Representative data is shown as mean ±SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated.

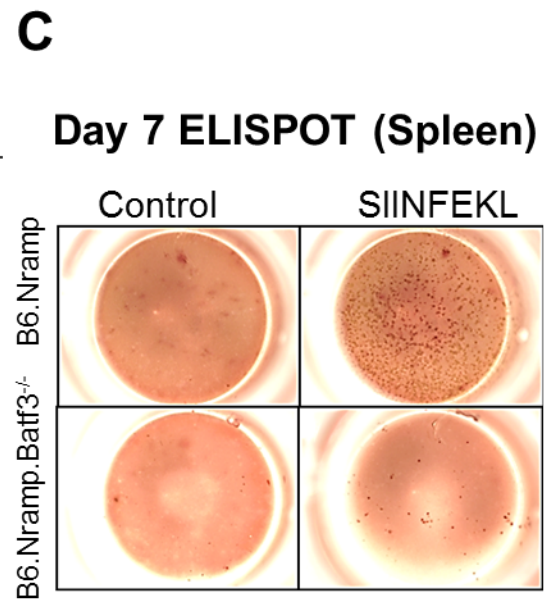
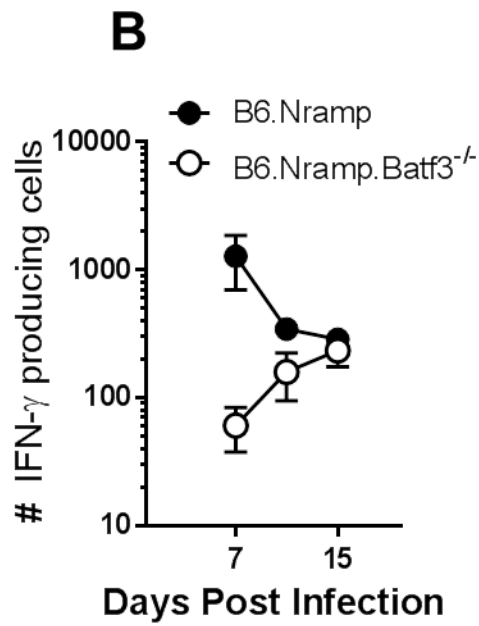
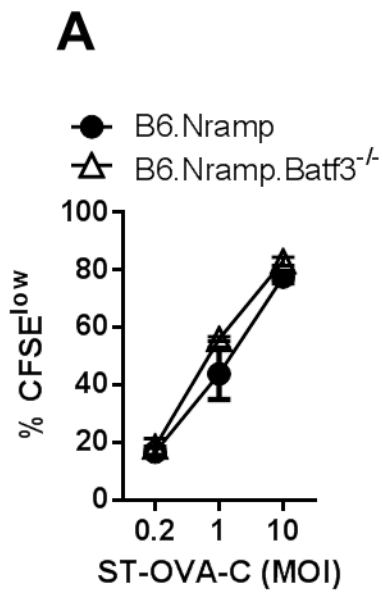
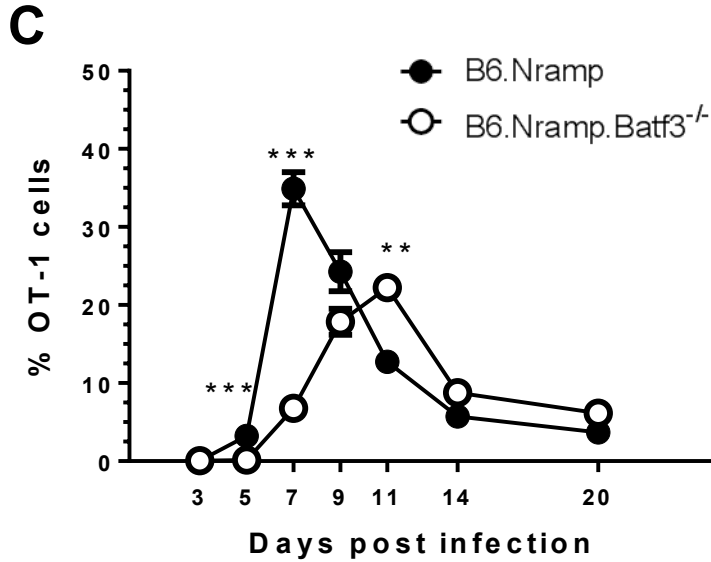
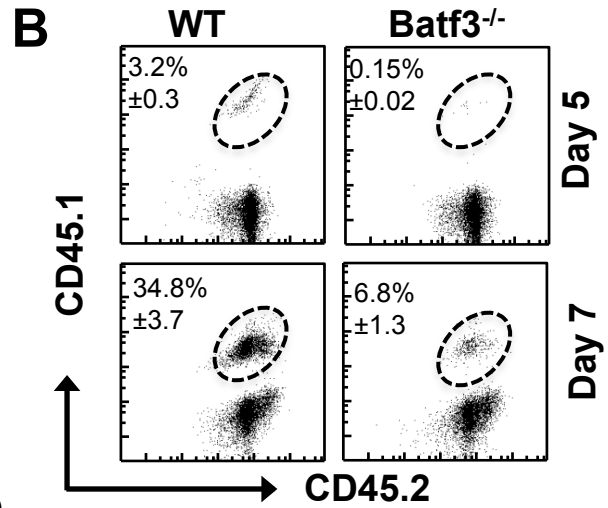
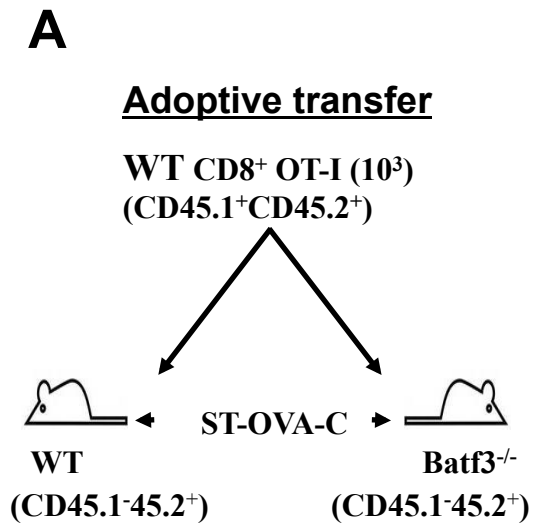


Figure 31: Delayed proliferation of OT-1 CD8⁺ T cells following ST-OVA-C infection in Batf3-deficient hosts

(A) B6.Nramp and B6.Nramp.Batf3^{-/-} mice (with endogenous cells expressing CD45.1⁻CD45.2⁺) were injected with WT OT-1 (CD45.1⁺CD45.2⁺) CD8⁺ T cells (10⁵ *i.v.*). Two days later, mice were infected with ST-OVA-C (10³ CFU *i.v.*). **(B)** At various time intervals, blood was collected from mice using saphenous bleeding and assessed for the numbers of proliferating OT-1 cells by flow cytometry. **(C)** Representative data is shown as mean ±SEM from 3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by student t test (**p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

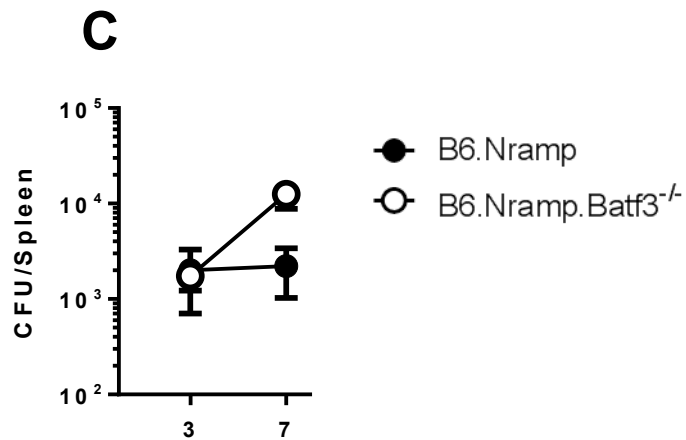
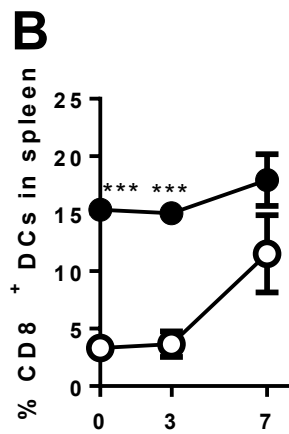
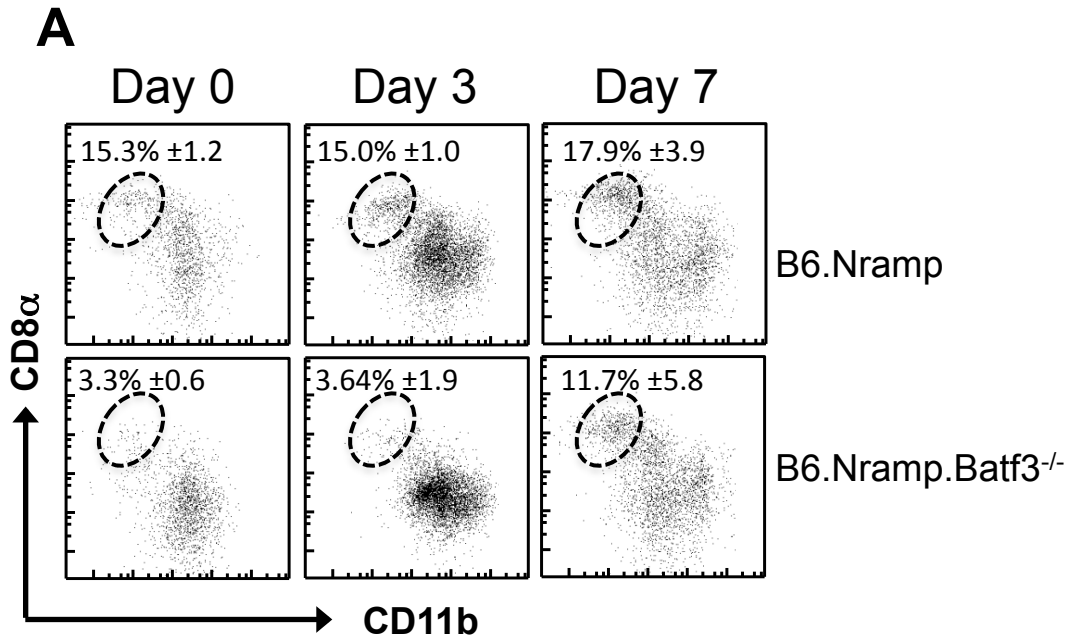


3.2.3 CD8⁺ T cell response in Batf3-deficient mice correlates with the number of CD8 α DCs

Batf3 is a critical transcription factor for the development of CD8 α DCs during steady state (101). Interestingly, under inflammatory conditions mediated by a pathogenic challenge, Batf3 independent mechanisms of CD8 α DC development are employed that utilize other transcription factors, which include Batf1 and Batf2 (379, 381). To assess the impact of such mechanisms, I evaluated the kinetics of CD8 α DCs in wild type and Batf3-deficient mice following infection with ST-OVA-C. During steady state and early stages of infection, the numbers of CD8 α DCs were greatly reduced in Batf3-deficient mice (**Fig. 32A, B**). However, Batf3-deficient mice developed a population of CD8 α DCs that emerged following 7 days of infection (**Fig. 32A, B**). Furthermore, Batf3-deficient mice had higher bacterial burden at later stages of infection (**Fig. 32C**). The delayed kinetics of the subsequent CD8⁺ T cell response against ST-OVA-C in Batf3-deficient mice correlated with the re-emergence of CD8 α DCs following the inflammation induced by higher burden of ST that emerges during later stages of infection (**Fig 32A-C**). These data indicate that the delayed CD8⁺ T cell response measured in Batf3-deficient mice following ST-OVA-C challenge (**Fig. 30, 31**) is mediated by the re-emergence of CD8 α DCs following ST challenge (**Fig 32A-B**). Furthermore, impaired CD8⁺ T cell priming in Batf3-deficient mice had a great impact on host survival when OVA antigen was translocated to the cytosol, which is only apparent in the Nramp mouse model due to prolonged survival of wild type mice (**Fig. 33**).

Figure 32: CD8 α DCs re-emerge in Batf3-deficient mice following ST infection

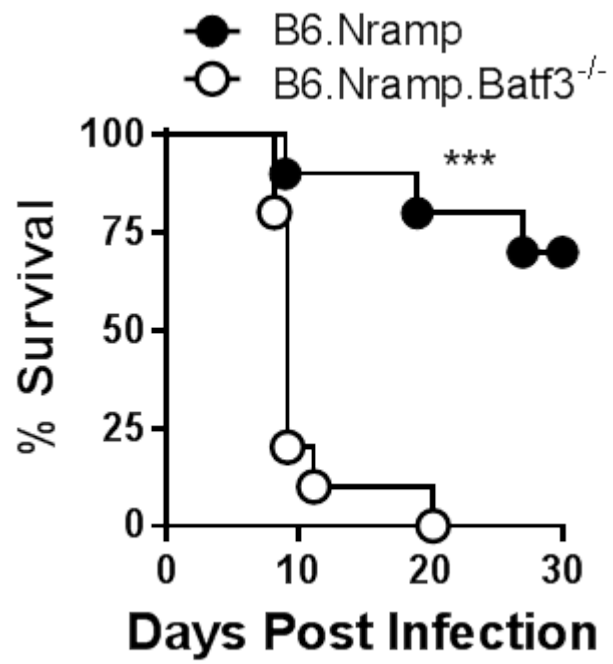
(A-B) B6.Nramp and B6.Nramp.Batf3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*) and assessed for splenic CD8 α and CD11b DC populations at day 0, 3 and 7 following infection. Splenic bacterial burden was also assessed on various time points following infection. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by student t test (**p<0.001).



Days post-infection

Figure 33: Batf3 impacts host survival against recombinant ST-OVA-C

Wild type and Batf3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*) and assessed for host survival. Representative data is shown as mean ±SEM from 2 experiments. Statistical significance on survival data was determined using Gehan-Breslow-Wilcoxon tests (**p<0.001).



3.2.4 Batf3-dependent DCs are critical to mount a CD8⁺ T cell response against ST in the draining lymph nodes

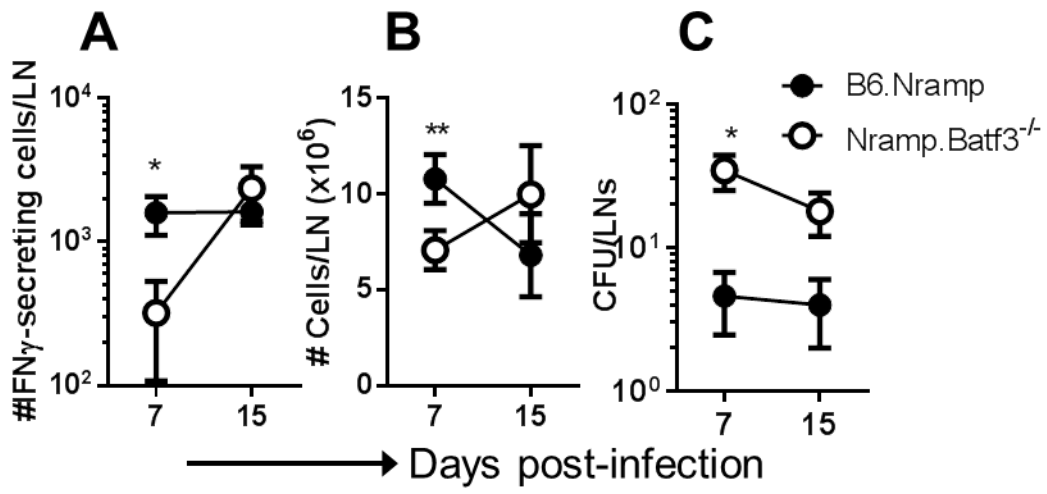
Thus far, my data suggests that Batf3-dependent DCs are critical in mounting a CD8⁺ T cell response against a systemic challenge model of ST (intravenous infection). However, the impact on localized ST challenge, which employs Batf3-dependent migratory CD103⁺ DCs and CD8 α DCs in the draining lymph nodes, was unclear. To address this issue, I utilized a localized challenge model of ST and injected ST-OVA-C subcutaneously. CD8⁺ T cell response was assessed in the draining inguinal lymph nodes at days 7 and 15 post- infection, the (Fig. 34A). CD8⁺ T cell response in the draining lymph nodes was poor in Batf3-deficient mice at day 7, but not day 15 post-infection (Fig. 34A, D). Reduced CD8⁺ T cell response in Batf3-deficient mice correlated with lower influx of cells in the lymph nodes (Fig. 34B), as well as higher bacterial burden (Fig. 34C). Interestingly, bacterial burden was significantly higher in Batf3-deficient mice, which indicates that ST is able to traffic to the draining lymph nodes independent of CD8 α DCs. However, delayed CD8⁺ T cell kinetics may also contribute to higher bacterial burden (Fig. 34A, C). These results suggest that Batf3-dependent DCs are critical for priming CD8⁺ T cell following infection in both systemic and localized challenge models of ST.

3.2.5 Batf3-dependent DCs are a critical source of inflammatory cytokines and chemokines during ST infection

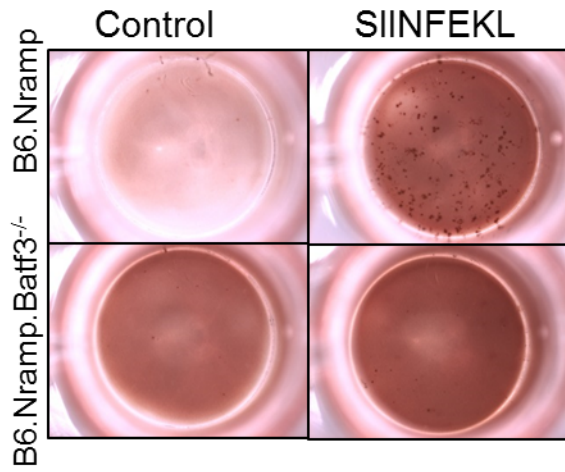
Different DC subsets express a variety of PRRs and thus have varying capabilities to respond to an array of pathogens, which may also impact antigen presentation and CD8⁺ T cell priming. For instance, cytokines and chemokines

Figure 34: Batf3 promotes early CD8⁺ T cell priming in a localized ST infection model

B6.Nramp and B6.Nramp.Batf3^{-/-} mice were infected with ST-OVA-C (10⁴ s.c.) at the base of the tail. Following 7 and 15 days of infection, the draining (inguinal) lymph nodes were harvested and assessed for **(A, D)** OVA₂₅₇₋₂₆₄-specific, IFN- γ producing cells by ELISPOT assay, **(B)** total numbers of cells in the lymph nodes, and **(C)** bacterial burden in the combined inguinal lymph nodes. Representative data is shown as mean \pm SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by student t test (*p<0.01, **p<0.01).



D Day 7 ELISPOT (dLN)



can also be critical in priming CD8⁺ T cells against pathogens by providing signal 3, which promotes migration and proliferation of primed cytotoxic T cells against ST. Splenic Nramp and Nramp.Batf3-deficient DCs were infected with ST *in-vitro* and the expression of various cytokines and chemokines was evaluated from the supernatants collected 24 hours after infection. Interestingly, chemokines that are important for T cell recruitment such as MIP-1 α and MIP-1 β were significantly diminished in Batf3^{-/-} DCs following ST infection (**Fig. 35A**). Batf3-dependent DCs expressed higher levels of inflammatory cytokines, such as IL-1 α , IL-6 and TNF- α following ST infection (**Fig. 35B**). Anti-inflammatory cytokine IL-10 had no dependency on CD8 α DCs (**Fig. 35B**). I also generated DCs from the bone marrow of wild type and Batf3-deficient mice and noted a similar impairment in cytokine production in Batf3-deficient GM-CSF-DCs as compared to wild type (**Fig. 36**). These results suggest that Batf3-dependent CD8 α DCs promote the expression of inflammatory cytokines and chemokines following ST challenge, which impacts priming of CD8⁺ T cells *in-vivo*.

Overall my data suggests that Batf3-dependent DC populations such as CD8 α DCs and CD103⁺ DCs, are critical for immune protection against ST, mediated by priming CD8⁺ T cells in both systemic and localized challenge model. Furthermore, this impact was observed in both recombinant strains of ST that induce either cross-presentation or direct-presentation. This indicates that, CD8 α DCs and CD103⁺ DCs also provide a critical source of essential cytokines and chemokines that are required for protective immunity against ST irrespective of antigenic location within the infected APC (**Fig. 37**).

Figure 35: Reduced production of inflammatory cytokines and chemokines in Batf3-deficient DCs following ST infection

DCs were infected with ST at various MOIs for 30 min, followed treatment with gentamycin for 2 hours. 24 hours following infection, the supernatant was collected and the expression of **(A)** chemokines: MIP-1 α , MIP-1 β , RANTES and **(B)** cytokines: TNF α , IL-6, IL-10 and IL-1 α was evaluated. Representative data is shown as mean \pm SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

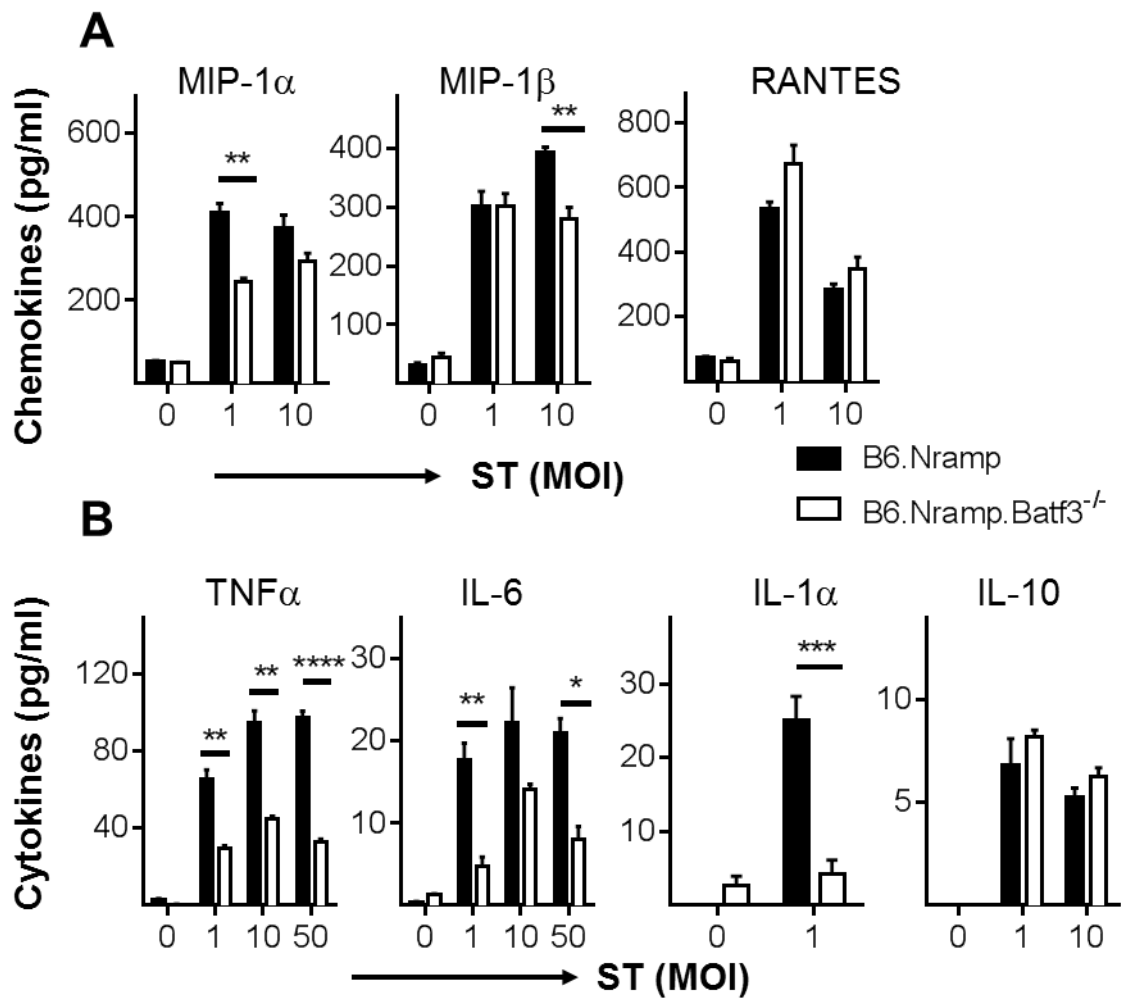


Figure 36: Impaired cytokine production in Batf3-deficient bone marrow derived cells following ST infection

GMCSF-DCs were plated into a 96-well plate and infected with ST at various MOIs, followed by a 2 hour treatment of gentamycin to eliminate extracellular bacteria. Following 24 hours post-infection, supernatants were collected and analyzed for cytokine secretion of IL-1 α , IL-1 β , IL-6 and TNF- α . Representative data is shown as mean \pm SEM from 2-3 experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01).

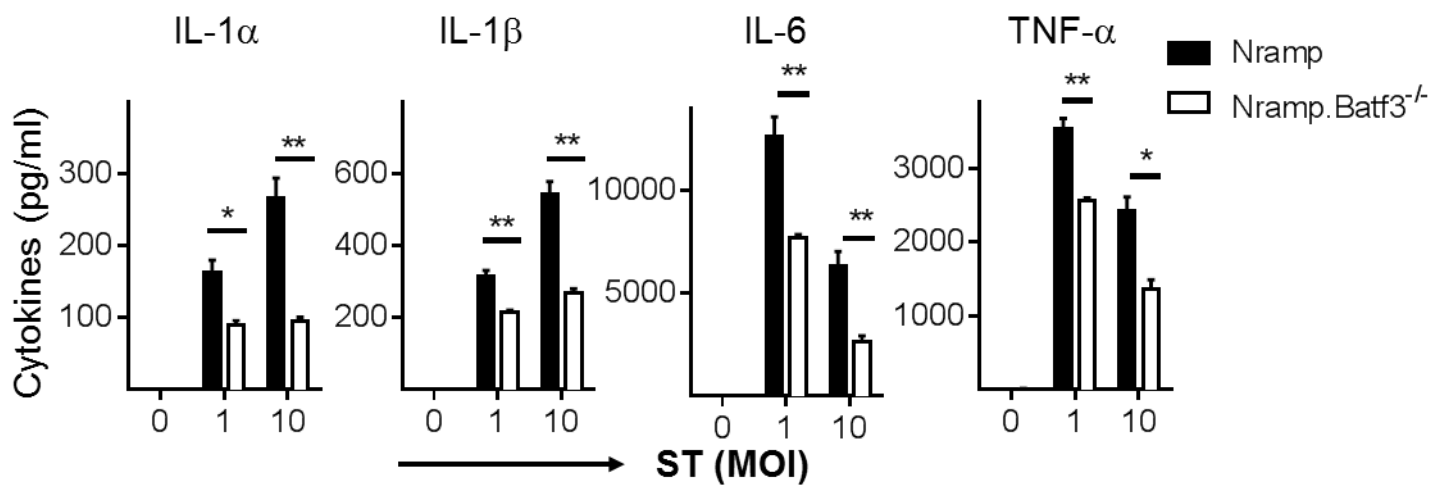
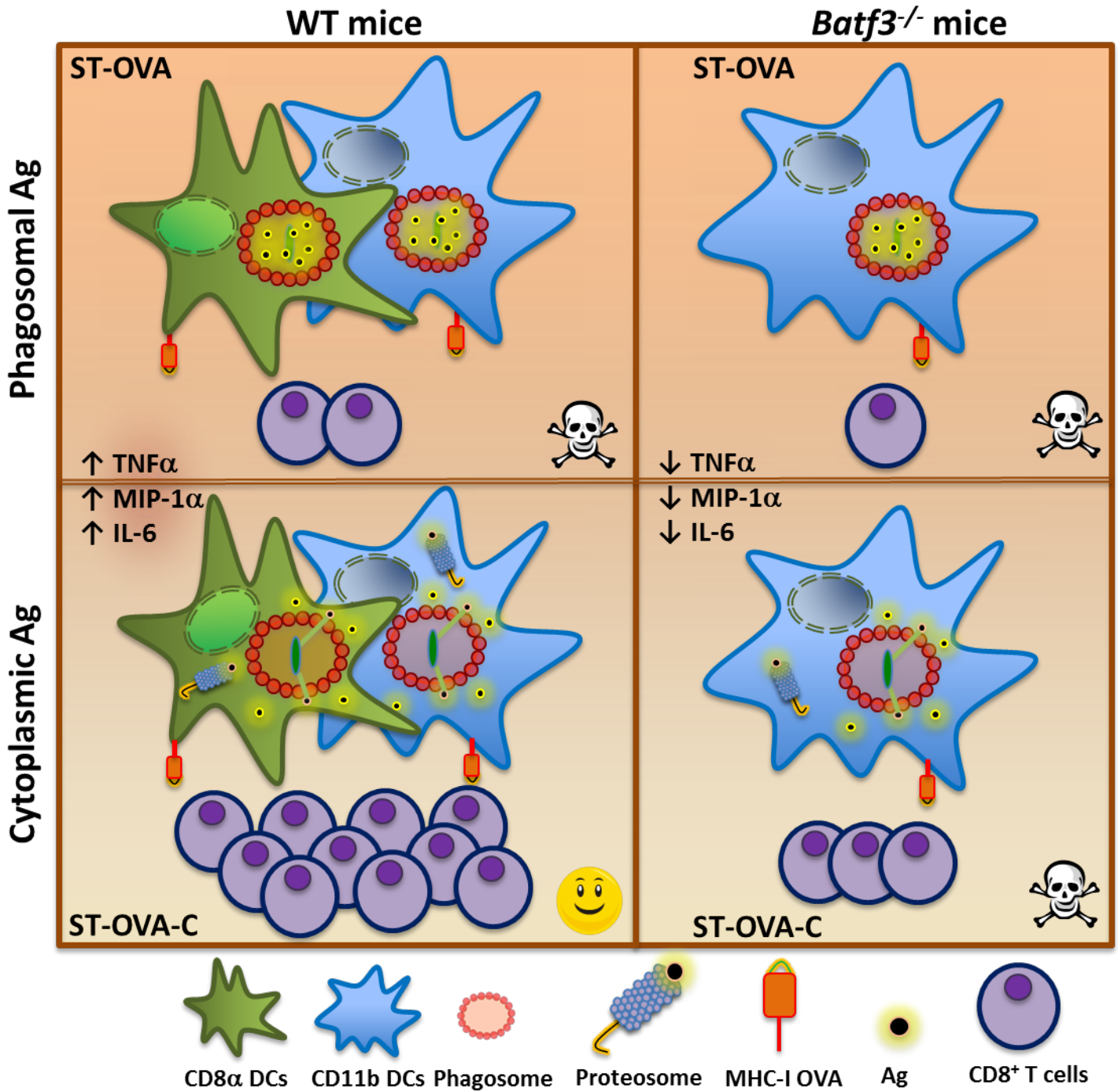


Figure 37: Visual Abstract II: Batf3-dependent DCs are required for immune protection against ST independent of the location of immunogenic antigen

Batf3 is a critical transcription factor that is required for proper development of CD8 α and CD103⁺ DCs at steady state. By utilizing recombinant strains of ST, that either deposit model antigen in the phagosome (ST-OVA) or cytoplasm (ST-OVA-C) of infected cells, I was able to determine the critical role of Batf3-dependent CD8 α and CD103⁺ DCs against ST infection. Interestingly, Batf3^{-/-} mice succumb to infection with a high dose of ST-OVA-C. Furthermore, production of inflammatory cytokine such as TNF- α , MIP-1 α and IL-6 requires Batf3-dependent DCs following infection with ST recombinants. Collectively, our data suggests that Batf3-dependent DCs are required to mediate a CD8⁺ T cell response against ST recombinants, critical for comprehensive immune protection and host survival. Batf3-dependent DCs are critical for host survival against the recombinant ST-OVA-C, which maintains antigen in the cytoplasm, indicating that the requirement for CD8 α DCs is independent of their ability for cross-presentation.



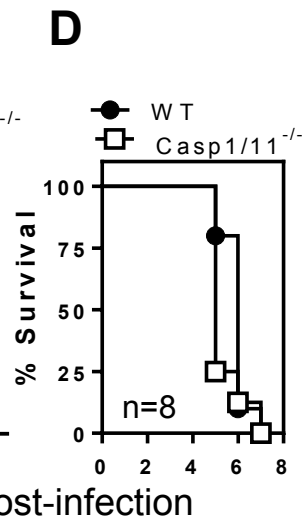
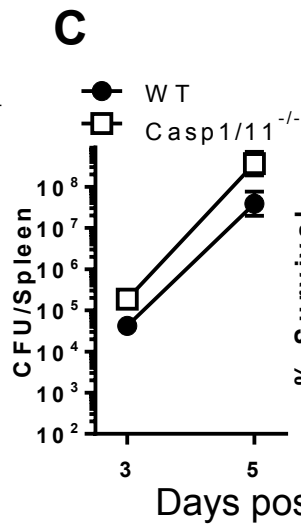
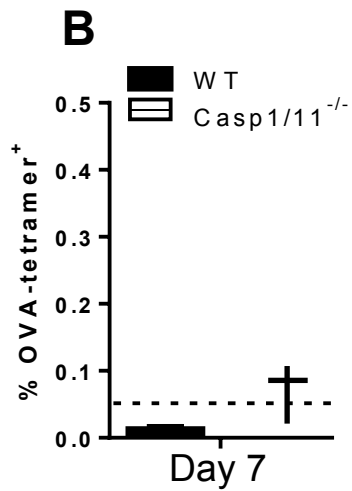
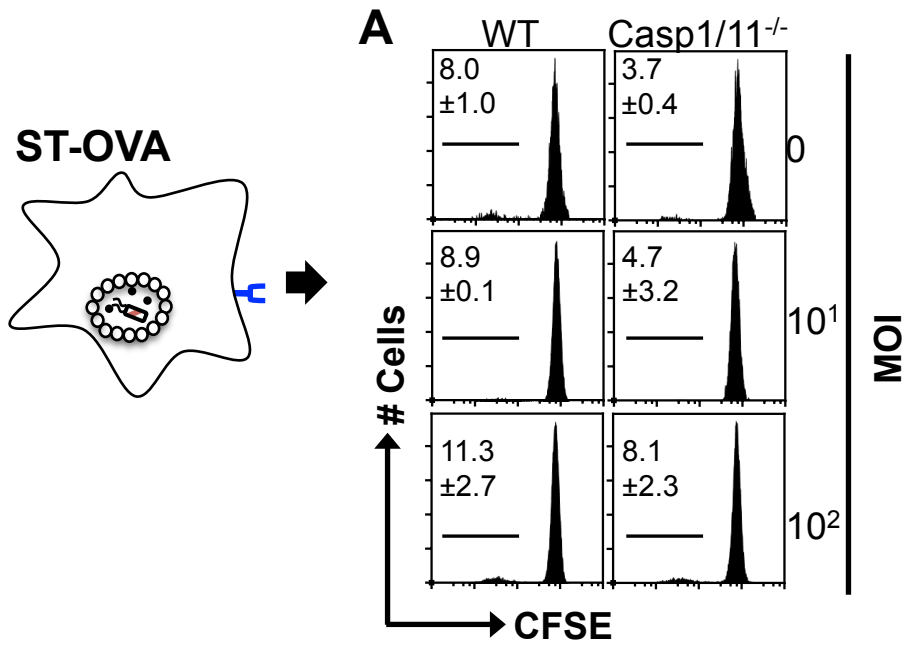
3.3 CHAPTER 3: Synergism of Caspase1/11 and RipK3 signalling pathways in control of intracellular bacteria

3.3.1 Early antigen presentation is necessary to reveal the protective role of inflammatory cell death pathways

Virulent ST induced rapid death of infected cells, and it is not clear what influence the rapid cell death of infected cells has on the development of CD8⁺ T cell response. Death of infected cells in various infection models has been shown to promote uptake of apoptotic bodies by surrounding DCs, which promotes cross-presentation of antigen (94, 113, 382). However, death of infected macrophages and DCs following infection with ST has been shown to be mediated mainly by inflammasome signalling, which results in cell death by pyroptosis, a pathway distinct from apoptosis (225). Caspase1/11 is a critical component of the inflammasome, which mediates the processing of IL-1 family of cytokines and cell death by pyroptosis, which results in release of intracellular DAMPs and exacerbates inflammation. Since pyroptosis is the dominant mechanism of cell death following ST infection (224, 383), I evaluated whether pyroptotic death in APCs modulates CD8⁺ T cell priming. I infected wild type and Caspase1/11-deficient DCs with ST-OVA, which expresses antigen in phagosomes (**Fig. 38A**). Wild type DCs as expected failed to induce early antigen presentation due to phagosomal location of antigen. Interestingly, there was no induction of antigen presentation with Caspase1/11-deficient DCs (**Fig. 38A**), implying that premature death of infected DCs is not the reason for the lack

Figure 38: Caspase1/11 signalling provides minimal impact on immune protection against phagosomal ST infection

ST-OVA is a recombinant strain of ST (SL1344) that deposits OVA into the phagosome of infected cells. **(A)** WT and Caspase1/11^{-/-} CD11c cells were plated into a 96-well plate and infected with ST-OVA at various MOIs, followed by gentamycin treatment for 2 hours to remove any extracellular bacteria. Cells were then incubated with CFSE labeled OT-1 transgenic (OVA₂₅₇₋₂₆₄ specific) CD8⁺ T cells. After 72 h, CFSE-dilution (indicative of proliferation) was assessed by flow cytometry on the CD8⁺ T cells. **(B)** WT and Caspase1/11^{-/-} mice were infected with ST-OVA (10³ CFU *i.v.*). Numbers of OVA-specific CD8⁺ T cells in the spleens were evaluated by OVA-tetramer staining at day 7. **(C)** Splenic bacterial burden and **(D)** host survival was evaluated. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. The cross symbol indicates that mice were euthanized before the time-point assessed was reached. All data points represent an n=3 unless otherwise stated.

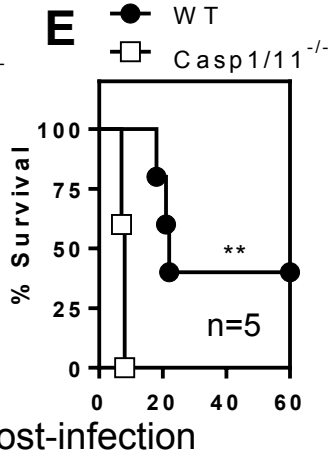
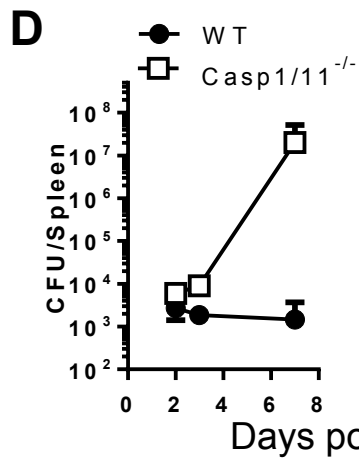
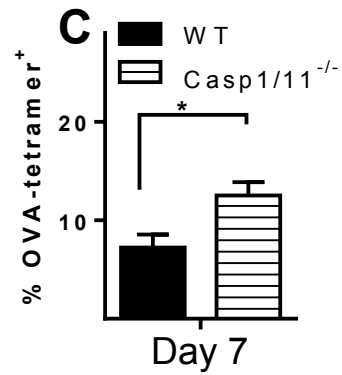
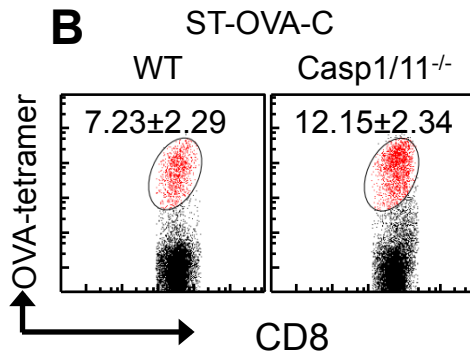
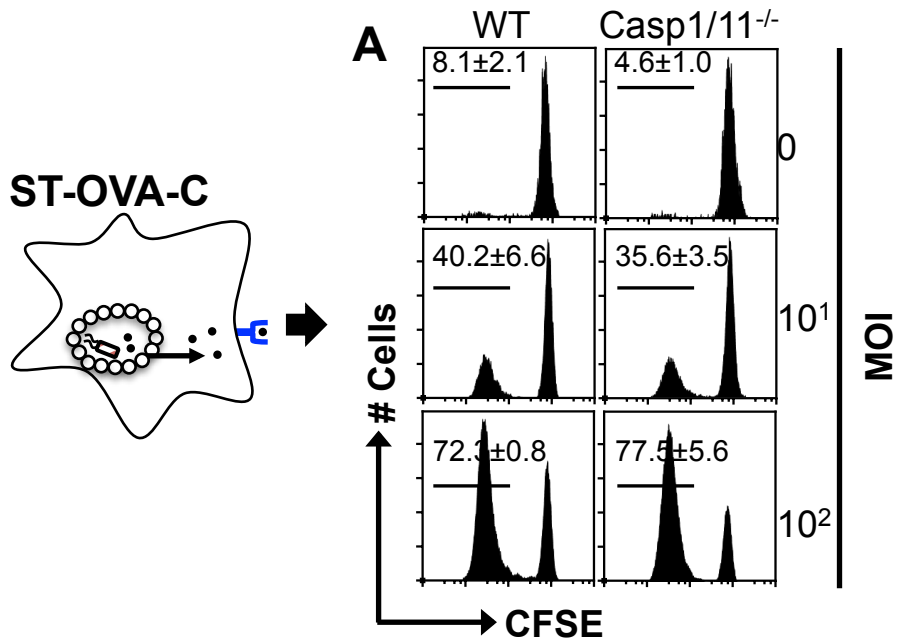


of detectable antigen presentation early during infection. Following intravenous infection with ST-OVA in wild type and Caspase1/11-deficient mice, there was no detectable CD8⁺ T cell response at day 7 (**Fig. 38B**), and there was a slight increase in the bacterial burden of Caspase1/11-deficient mice at all the time points tested (**Fig. 38C**). However, this had no implication on host survival as both groups of mice succumbed to infection at similar rates (**Fig. 38D**).

Recombinants of ST that express antigen in the phagosome induce a delayed CD8⁺ T cell response (161). In contrast, ST-OVA-C is a recombinant that translocates antigen into the cytoplasm and engages proteasomal antigen processing (46). As a result ST-OVA-C induces a robust CD8⁺ T cell response within 7 days of infection (46). I tested the impact that Caspase1/11 has on antigen presentation, host survival and priming CD8⁺ T cell responses against this recombinant strain of ST. As expected, ST-OVA-C induced robust antigen presentation *in-vitro* (**Fig. 39A**). Interestingly, OT-1 proliferation was similar between wild type and Caspase1/11-deficient DCs infected with ST-OVA-C at various MOIs, indicating that Caspase-1/11 has little impact on the ability of DCs to engage antigen presentation (**Fig. 39A**). The endogenous CD8⁺ T cell response measured at day 7 was significantly higher in Caspase1/11-deficient mice (**Fig. 39B-C**), which correlated with increased bacterial burden, up to 1000 fold higher than wild type mice (**Fig. 39D**). Increased bacterial burden at day 7 in Caspase1/11-deficient mice (**Fig. 39D**) may be the reason I observe a stronger endogenous CD8⁺ T cell response in comparison to wild type mice (**Fig. 39B-C**). Furthermore, Caspase1/11-deficient mice succumbed to infection rapidly, in

Figure 39: Engaging early antigen-presentation reveals the protective role of Caspase1/11 signalling against ST infection

ST-OVA-C is a recombinant strain of ST (SL1344) that deposits OVA into the cytosol of infected cells. **(A)** WT and Caspase1/11^{-/-} CD11c cells were plated onto a 96-well plate and infected with ST-OVA-C at various MOIs. Extracellular bacteria were removed by gentamicin treatment, and cells were incubated with CFSE labeled OT-1 transgenic (OVA₂₅₇₋₂₆₄ specific) CD8⁺ T cells. After 72 h, CFSE-dilution (indicative of proliferation) was assessed by flow cytometry on the CD8⁺ T cells. **(B-C)** WT and Caspase1/11^{-/-} mice were infected with ST-OVA-C (10³ CFU i.v.). Numbers of OVA-specific CD8⁺ T cells in the spleens were evaluated by OVA-tetramer staining at day 7. **(D)** Splenic bacterial burden and **(E)** host survival was evaluated. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05) or the Gehan-Breslow-Wilcoxon test for survival data (**p<0.01).



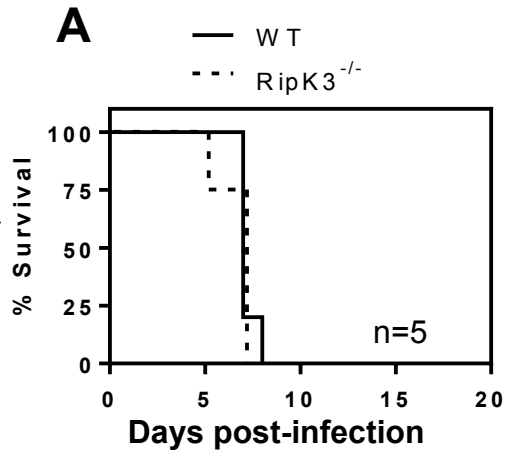
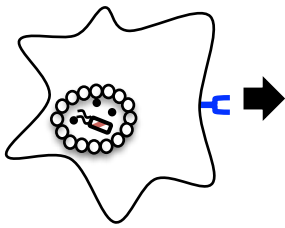
comparison to wild type mice, some of which survived the course of infection (**Fig. 39E**).

Necroptosis is an inflammatory form of cell death that is engaged by sentinel immune cells during ST infection and may have implications on antigen presentation and host survival. RipK3 is the critical kinase that mediates cell death by necroptosis (229, 269). Thus, I proceeded to analyze the impact of RipK3 dependent necroptosis on CD8⁺ T cell response during infection with ST-OVA and ST-OVA-C recombinants of ST. Wild type and RipK3-deficient mice rapidly succumbed to infection following infection with ST-OVA (**Fig. 40A**). Accordingly, bacterial burdens in both mice were similar at time points tested (**Fig. 40B**). As expected, endogenous CD8⁺ T cell responses were undetectable in both wild type and RipK3-deficient mice (**Fig. 40C**). These results indicate that RipK3 signalling pathway of cell death is not responsible for the delayed CD8⁺ T cell response observed against ST-OVA. Interestingly, challenging wild type and RipK3-deficient mice with ST-OVA-C revealed that RipK3 signalling promotes host survival when antigen presentation is engaged early during infection (**Fig. 40D**). Enhanced fatality in RipK3-deficient mice correlated with increased bacterial burden (**Fig. 40E**). CD8⁺ T cell response against ST-OVA-C was higher in RipK3-deficient mice than wild type, possibly as a consequence of higher bacterial burden observed in Caspase1/11-deficient mice (**Fig. 40F**).

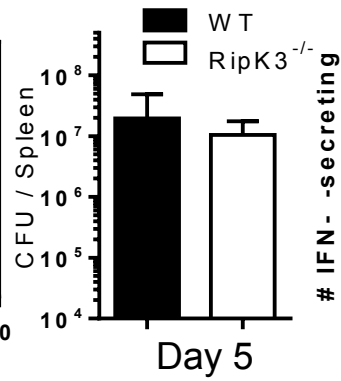
Figure 40: RipK3 plays a protective role only when antigen-presentation is engaged early during infection

WT and RipK3^{-/-} mice were infected with ST-OVA (10³ CFU *i.v.*) and assessed for **(A)** host survival and **(B)** bacterial burden at day 5 post infection. **(C)** 7 days following infection, OVA-specific CD8⁺ T cell response against ST-OVA was evaluated in the spleens of infected mice by ELISPOT assay. Similarly, wild type and RipK3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*) and assessed for **(D)** host survival and **(E)** bacterial burden at day 5 post infection. **(F)** OVA-specific CD8⁺ T cell response against ST-OVA-C was evaluated in the spleens of infected mice at day 7 by ELISPOT assay. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by Gehan-Breslow-Wilcoxon test for survival data (**p<0.01).

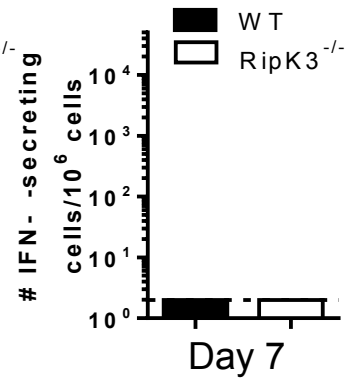
ST-OVA



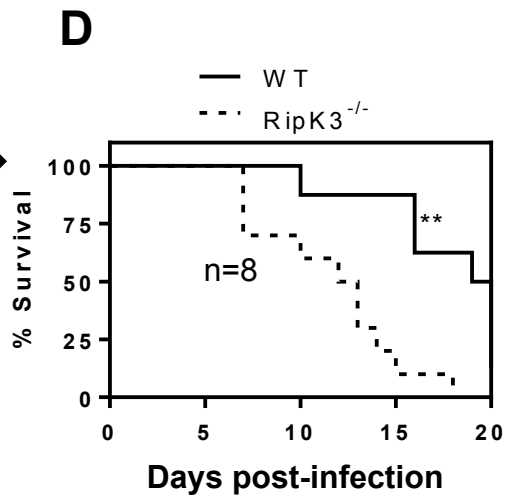
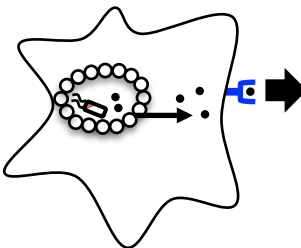
B



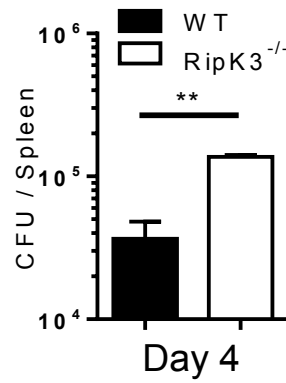
C



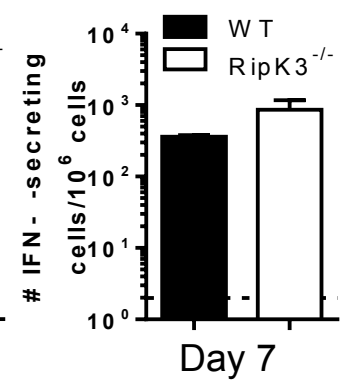
ST-OVA-C



E



F



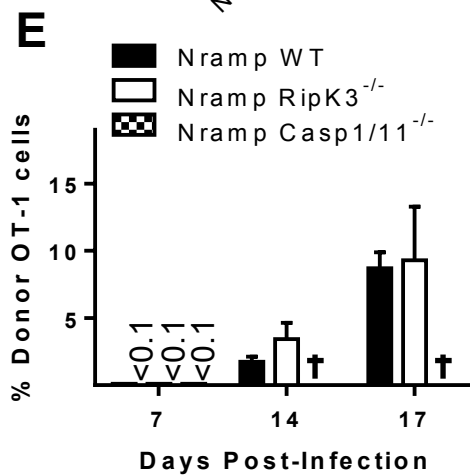
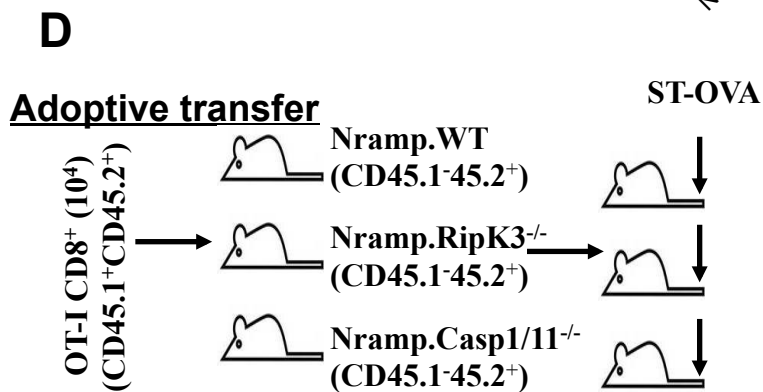
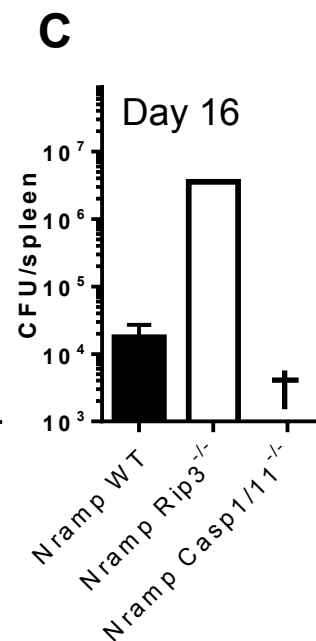
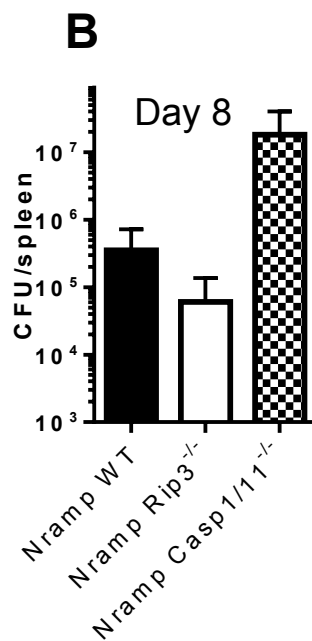
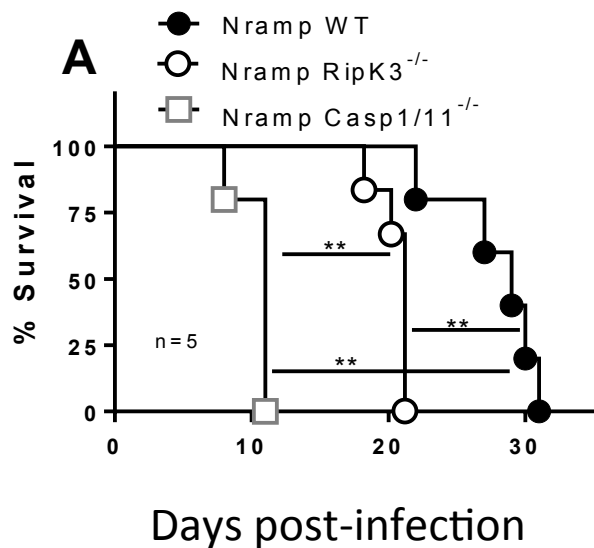
3.3.2 Expression a functional *Nramp1* gene reveals the critical role of Caspase1/11- and RipK3-signalling during ST infection

I did not observe any significant differences in host survival between wild type mice and Caspase1/11- or RipK3-deficient mice following infection with ST-OVA, however ST-OVA-C infection yielded significant differences (**Fig. 38-40**). Since ST-OVA induces rapid fatality in susceptible mice, this may not allow sufficient time to reveal the protective roles of Caspase1/11 and RipK3 during infection with ST-OVA. Rapid fatality following ST infection is partly attributed to a non-functional *Nramp1* gene expressed by susceptible mice (179). I therefore tested the impact of Caspase1/11 and RipK3 against ST-OVA in B6 mice expressing a functional *Nramp1* gene. I observed that *Nramp* mice deficient in either Caspase1/11 or RipK3 succumb to infection earlier than their wild type counterparts (**Fig. 41A**). Caspase1/11-deficient mice exhibited a significantly higher bacterial burden as compared to wild type and RipK3-deficient mice at day 8 post-ST-OVA infection (**Fig. 41B**). Furthermore, the bacterial burden was significantly higher in RipK3-deficient mice as compared to wild type mice at day 16 post-infection (**Fig. 41C**). Thus, these results indicate that even when antigen presentation is delayed, Caspase1/11 and RipK3 signalling promotes protection against infection when *Nramp1* gene is functional. Furthermore, results indicated that Caspase1/11 signalling has a greater impact than RipK3 on survival against ST (**Fig. 41A**).

To further evaluate the impact on CD8⁺ T cell response, I performed an adoptive transfer experiment by injecting OT-1 CD8⁺ T cells into recipient mice deficient in Caspase1/11 or RipK3 signalling followed by infection with ST-OVA

Figure 41: Expression of Nramp1 enhancing the survival of B6 mice reveals the critical role of Caspase1/11 and RipK3 signalling with phagosomal ST

B6.Nramp, B6.Nramp.RipK3^{-/-} and B6.Nramp.Caspase1/11^{-/-} mice were infected with ST-OVA (10³ CFU *i.v.*) and evaluated for **(A)** host survival and **(B)** bacterial burden at **(B)** day 8 and **(C)** day 16 following infection. **(D-E)** To assess the role of Caspase1/11 and RipK3 on antigen-presentation and CD8⁺ T cell priming against ST-OVA-C, we adoptively transferred OT-1 CD8⁺ T cells (CD45.1⁺CD45.2⁺) expressing a transgenic TCR that recognizes H2-K^b-OVA₂₅₇₋₂₆₄ into B6.Nramp, B6.Nramp.Caspase1/11^{-/-} and B6.Nramp.RipK3-deficient mice (CD45.1⁺CD45.2⁺) to assess for OT-1 proliferation from blood samples received by saphenous bleeding by flow cytometry collected at various days post-infection. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. The cross symbol indicates that mice were euthanized before the time-point assessed was reached. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by Gehan-Breslow-Wilcoxon test for survival data (**p<0.01). Experiments involving Caspase1/11-deficient mice in panel A-C were performed by Kristina Wachholz.



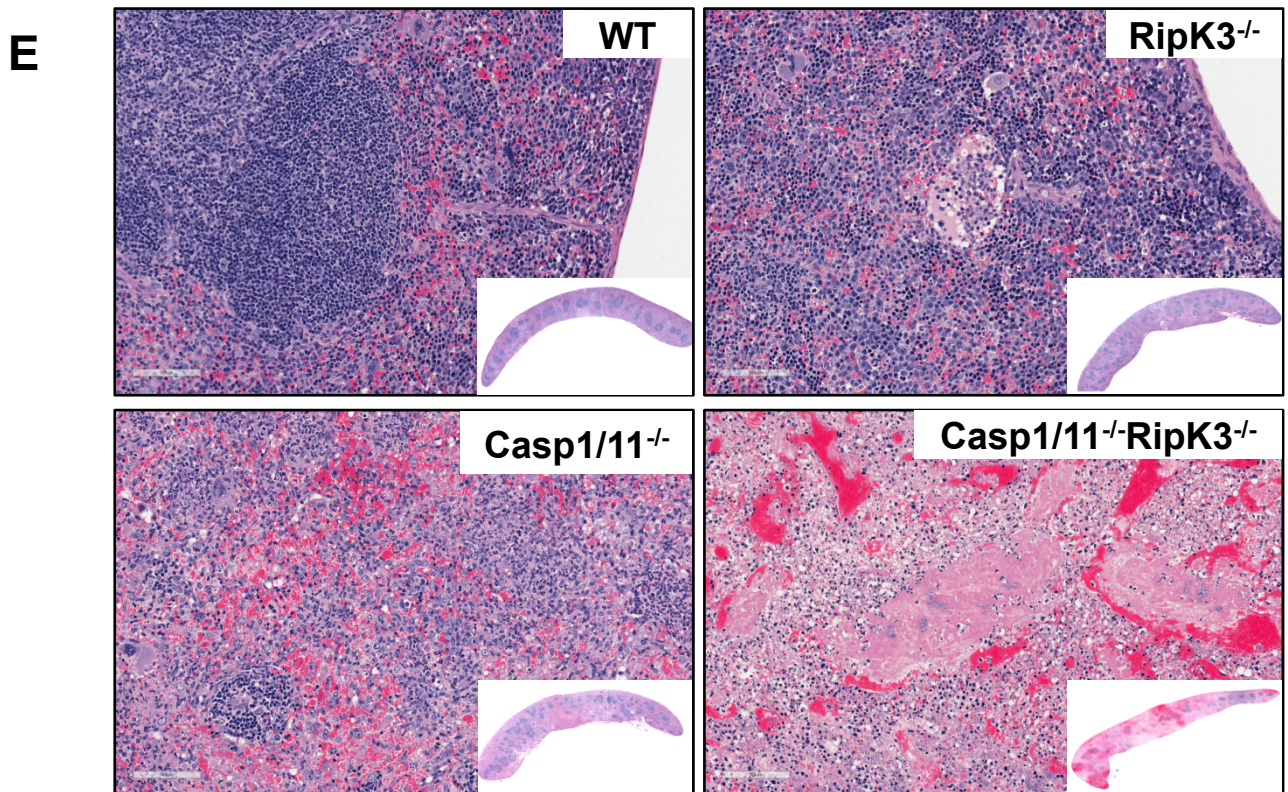
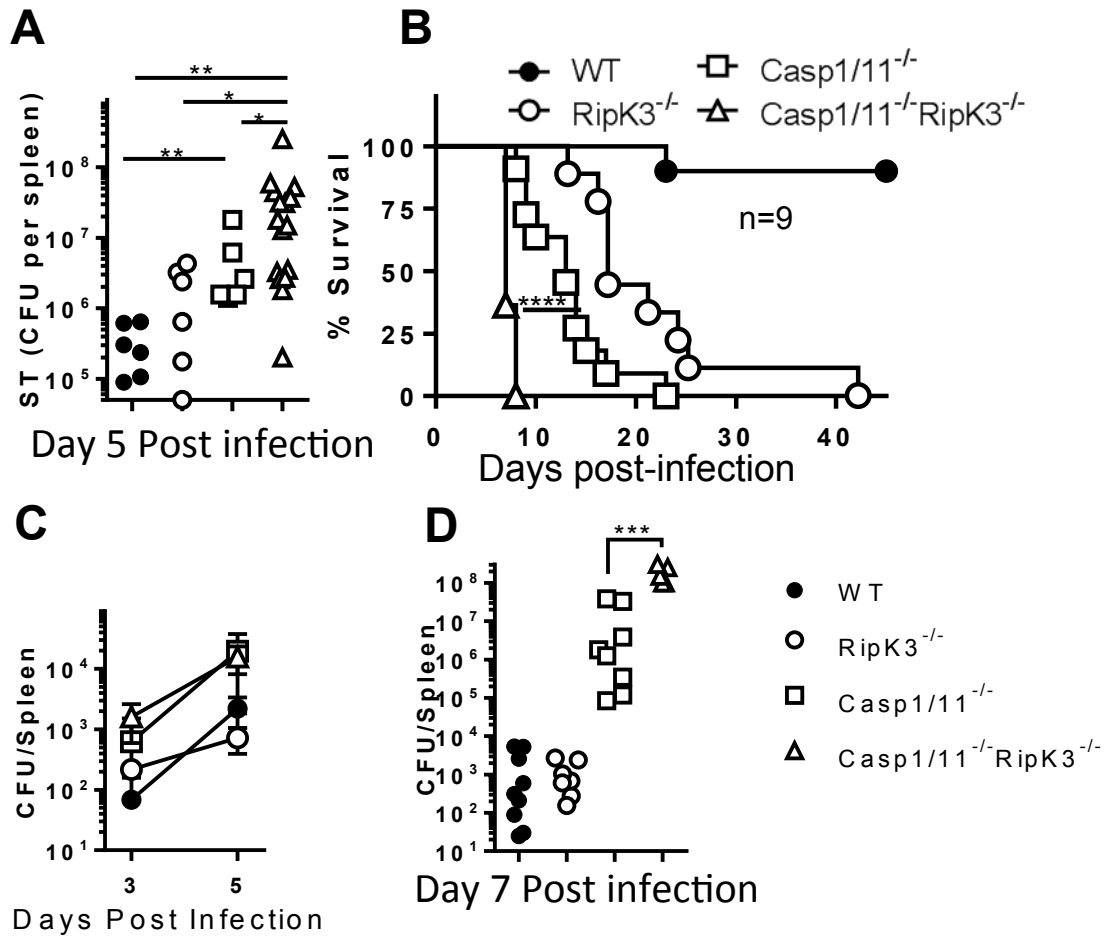
(**Fig. 41D**). There was no detectible CD8⁺ T cell response in wild type, Caspase1/11- or RipK3-deficient mice at day 7 post infection (**Fig. 41E**), indicating that delayed CD8⁺ T cell response against ST is not due to APCs engaging Caspase1/11 or RipK3 mediated pathways of cell death. As expected, Caspase1/11 succumbed to infection at an early time point (<10 days) (**Fig. 41E**). Following this time point, I did not observe any significant differences in CD8⁺ T cell priming among wild type or RipK3-deficient mice at all time-points measured (**Fig. 41E**). By utilizing the *Nramp1* transgenic model, I was able to recapitulate the dependence on Caspase1/11 and RipK3 on host survival against both ST-OVA and ST-OVA-C.

3.3.3 Disruption of both Caspase1/11 and RipK3 results in accelerated host fatality

Next I examined whether Caspase1/11 and RipK3 are complementary signalling pathways that promote death of infected cells and control of ST bacterial burden and whether this impacts CD8⁺ T cell response. To achieve this I created Caspase1/11 and RipK3 double deficient mice and infected them with ST (10^2 CFU *i.v.*). I observed a significant increase in bacterial burden in Caspase1/11-RipK3 double deficient mice as compared to Caspase1/11-deficient, RipK3-deficient or wild type mice (**Fig. 42A**). Due to early lethality of mice following infection with ST, I evaluated the impact in mice following infection with ST-OVA-C (10^3 CFU *i.v.*) and monitored bacterial burden and host survival. Caspase1/11-RipK3 double deficient mice succumbed to infection rapidly (< 7 days) following ST-OVA-C infection (**Fig. 42B**). In comparison, Caspase1/11-

Figure 42: Synergism of Caspase1/11 and RipK3 signalling during ST infection

(A) WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST (10³ CFU *i.v.*). At day 5 post infection, mice were sacrificed and splenic bacterial burden was assessed for each mouse group. WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*). (B) Each group was evaluated for host survival, as well as splenic bacterial burden at (C) day 3, day 5 and (D) day 7 post infection. (E) Spleens were harvested at day 7 post infection, treated with 10% formalin and vertical sections were stained for hematoxylin and eosin (H&E). Sections were analyzed and graded by a pathologist. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001). Histology sections were assessed and scored by Dr. Manijeh Daneshmand from the Pathology Department at the University of Ottawa.

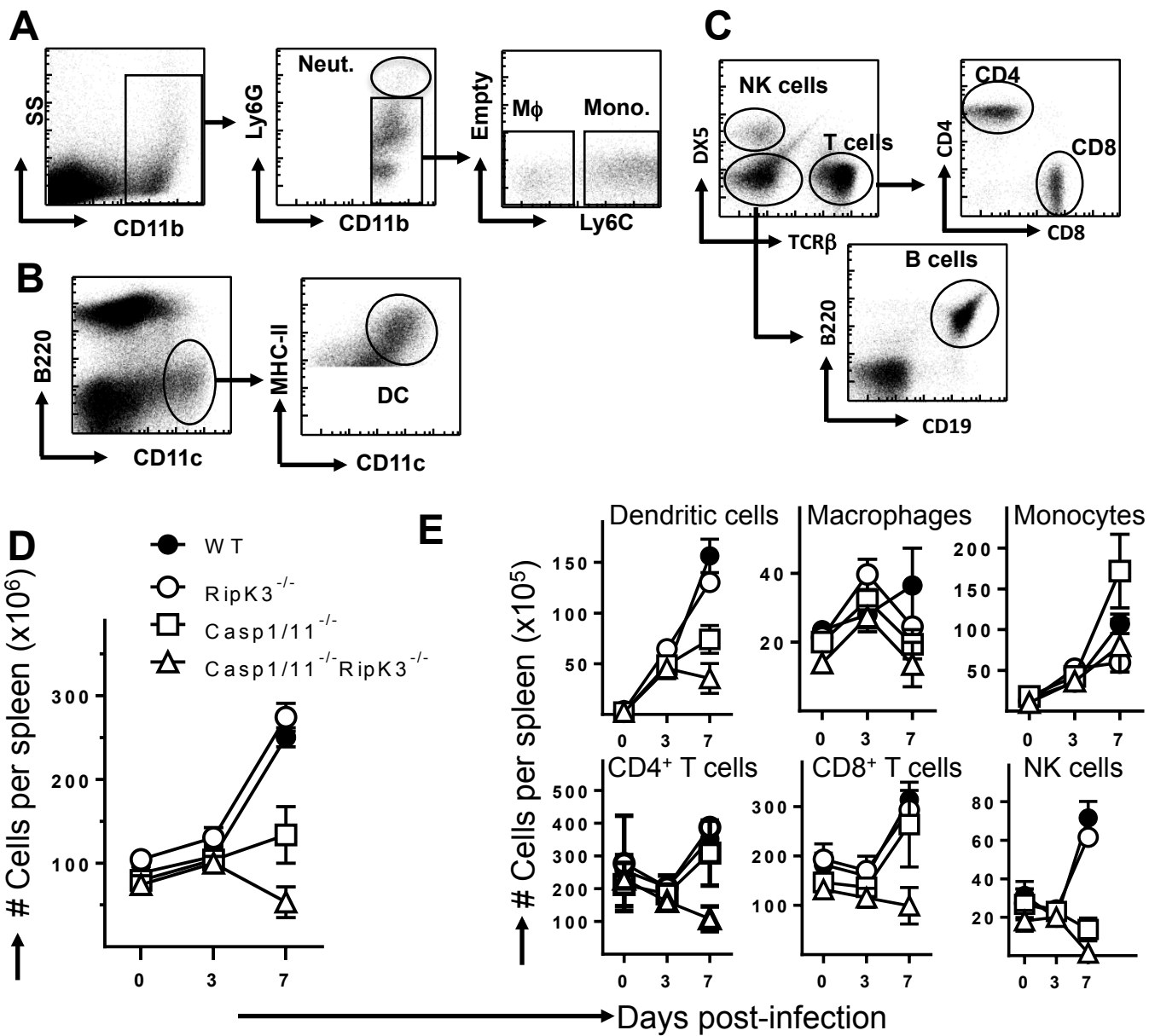


and RipK3-deficient mice exhibited increased mortality than wild type mice, at a median time of 15 and 20 days respectively (**Fig. 42B**). It was apparent that Caspase1/11 deficiency was detrimental against ST-OVA-C as Caspase1/11- and Caspase1/11-RipK3-deficient mice exhibited higher bacterial burden than wild type and RipK3-deficient mice at day 3 and day 5 following infection (**Fig. 42C**). Accordingly, the bacterial burden of Caspase1/11-RipK3-double deficient mice was significantly higher than all the other groups of mice at day 7 post-infection (**Fig. 42D**). Significant number of Caspase1/11-RipK3-deficient mice had to be sacrificed even before day 7, suggesting that the impact of double-deficiency is underestimated in **Fig. 42D**. These results indicate that both Caspase1/11 and RipK3 play a synergistic protective role against ST, which is more apparent at later stages of infection (**Fig. 42B-D**).

To further study the synergistic impact of Caspase1/11 and RipK3 signalling on protection against ST, I performed histological examination of spleen sections at day 7 post-infection and noted a graded impact on splenic pathology (Caspase1/11^{-/-}RipK3^{-/-} > Caspase1/11^{-/-} > RipK3^{-/-} > WT) (**Fig. 42E**). The splenic tissue in infected RipK3-deficient mice exhibited small foci of necrosis and hyperactive follicles (**Fig. 42E**). Caspase1/11-deficient mice exhibited increased signs of cell death with the white pulp being segmented and smaller (**Fig. 42E**). Finally, Caspase1/11-RipK3-deficient mice showed major signs of cell death and atrophy (wasting away) (**Fig. 42E**). At 7 days post infection, analysis using flow cytometry revealed elimination of various immune cell compartments in Caspase1/11-RipK3-double deficient mice (**Fig. 43A-E**) in addition to drastic

Figure 43: Diminished immune cell subsets following infection of RipK3, Caspase1/11 and double deficient mice with ST

WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*). Spleens were harvested and evaluated for different immune cell subset at various time points at day 0, 3 and 7 post-infection. **(A-C)** Representative gating strategies are shown to quantify neutrophils, DCs, monocytes, macrophages, NK cells, CD4⁺ T cells, CD8⁺ T cells and B cells. **(D-E)** Total cell numbers from harvested spleens as well as total number of immune cells was analyzed at various time points. All data points represent an n=3 unless otherwise stated. Data is shown as mean ±SEM and is representative of 2-3 separate experiments.



reduction in cell numbers (**Fig. 43D**). Surprisingly, disabling of Caspase1/11 and RipK3 dependent death pathways did not have an impact on CD8⁺ T cell responses against ST-OVA-C as wild type and Caspase1/11-RipK3-double deficient mice promoted a similar CD8⁺ T cell response 7 days post infection (**data not shown**).

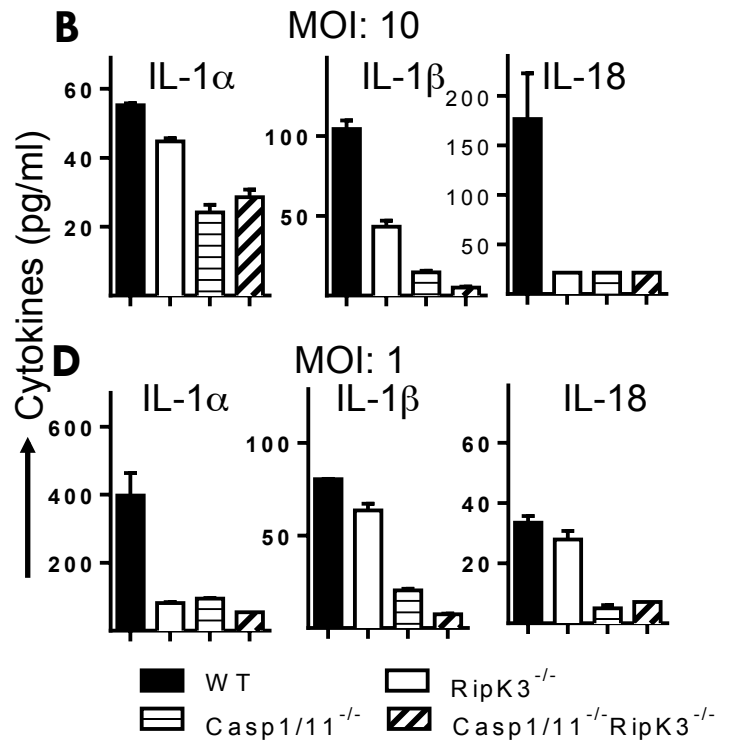
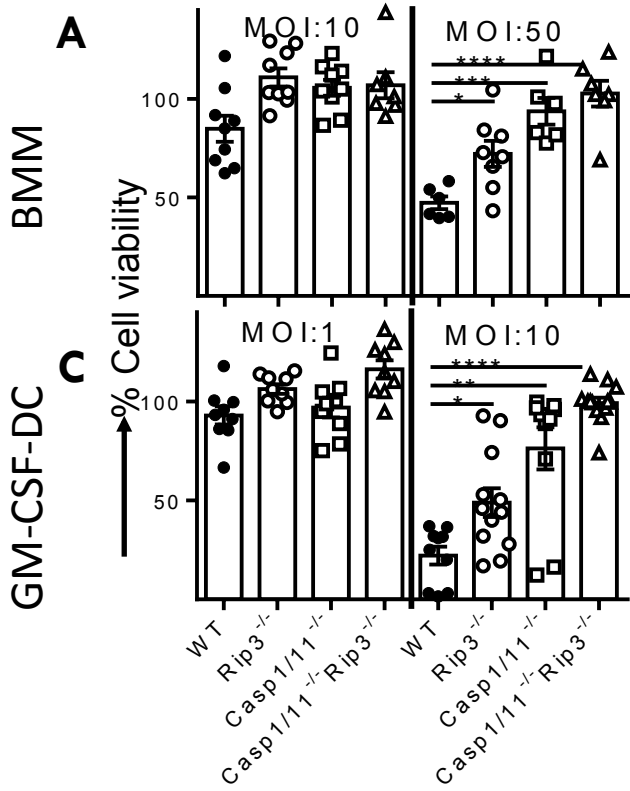
3.3.4 Synergistic impact of Caspase1/11 and RipK3 in inflammasome activation and cell-death

To further investigate the mechanism behind the synergistic impact of Caspase1/11 and RipK3 on control of ST, I tested cell death, activation, cytokine production in macrophages (BMMs) and DCs (GMCSF-DCs) *in-vitro*. I observed a graded impact on the ability of WT and knockout cells to engage cell death in BMM (**Fig. 44A**) and GMCSF-DCs (**Fig. 44C**) *in-vitro* (WT > RipK3^{-/-} > Caspase1/11^{-/-} > Caspase1/11^{-/-}RipK3^{-/-}) following 24 hours of infection with ST. Interestingly, GMCSF-DCs were more sensitive to ST induced cell death than BMMs. For instance, an MOI of 10 was required to induce potent cell death in wild type GMCSF-DCs (**Fig. 44C**). In comparison, an MOI of 50 was required to induce cell death in BMMs (**Fig. 44A**).

Next I examined cytokine production in both BMMs and GMCSF-DCs following ST infection. To ensure that differences in cell death did not impact cytokine production, I measured cytokine levels following infection at MOI levels that did not induce cell death in any of the groups tested (MOI of 1 for GMCSF-DCs and MOI of 10 for BMMs) (**Fig. 44A, C**). IL-1 inflammatory cytokine, such as IL-1 α and IL-1 β are considered to be DAMPs, which do not express a signal

Figure 44: Caspase1/11 and RipK3 are critical to mediate ST induced cell-death and inflammasome activation following infection of macrophages and DCs

BMMs (**A**) and GM-CSF-DCs (**C**) grown from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST for 30 minutes followed by a 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Following 24 hours post-infection, cells were evaluated for viability by neutral red assay. Additionally, supernatants were collected to assess for cytokine production from infected (**B**) macrophages and (**D**) DCs 24 hours following ST infection. Data is shown as mean ±SEM and is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



sequence for secretion through the Golgi, and thus are mainly released by dead or dying cells (240). Production of IL-1 α was greatly reduced in Caspase1/11, RipK3 and double deficient BMMs and GMCSF-DCs (**Fig. 44B, D**). Additionally, IL-1 β and IL-18 were also reduced in Caspase1/11- and RipK3-deficient BMMs and GMCSF-DCs (**Fig. 44B, D**). Defective cytokine production by Caspase1/11- and RipK3-deficient cells was specific to IL-1 cytokines as other inflammatory cytokines and chemokines were produced at normal levels (**Fig 45A, B**). IL-1 β levels were also reduced in BMMs and GMCSF-DCs stimulated with LPS *in-vitro* (**Fig. 46A, B**). Similarly, IL-1 β was lower in serum samples collected from Caspase1/11- and RipK3-deficient mice challenged with LPS for six hours (**Fig. 47**). In stark contrast, cytokine levels in serum collected from Caspase1/11- and RipK3-deficient mice had higher levels of pro-inflammatory cytokines following infection with ST-OVA-C, most likely due to enhanced bacterial burden, with the exception of IL-18, which was impaired in Caspase1/11- and Caspase1/11-RipK3-deficient mice (**Fig. 48**). I then examined the activation of NF- κ B and mitogen-activated protein kinase (MAPK) following ST infection in BMMs (**Fig. 49A**) and GMCSF-DCs (**Fig. 49B**), which is critical for promoting transcription of pro-IL1 α and pro-IL-1 β as part of the signal 1 for inflammasome activation (384). Caspase1/11 or RipK3 signalling did not have an impact on the activation of NF- κ B and MAPK as measured through phosphorylation of these signal mediators (p65 NF- κ B and p38 MAPK) (**Fig. 49A-B**). This data indicates that the impact of Caspase1/11 and RipK3 on IL-1 cytokine production is observed during

Figure 45: Induction of cytokine production in Caspase1/11- and RipK3-deficient macrophages and DCs following ST infection

(A) BMMs and (B) GM-CSF-DCs were grown from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST at an MOI of 10 for macrophages and 1 for DCs for 30 minutes to limit induction of cell death respectively. Cells were then washed and treated with gentamycin for 2 hours to eliminate any extracellular bacteria. Cells were incubated for 24 hours, at which point the supernatants were collected and assessed for cytokine production. All data points represent an n=3 unless otherwise stated. Data is shown as mean ±SEM and is representative of 2 separate experiments.

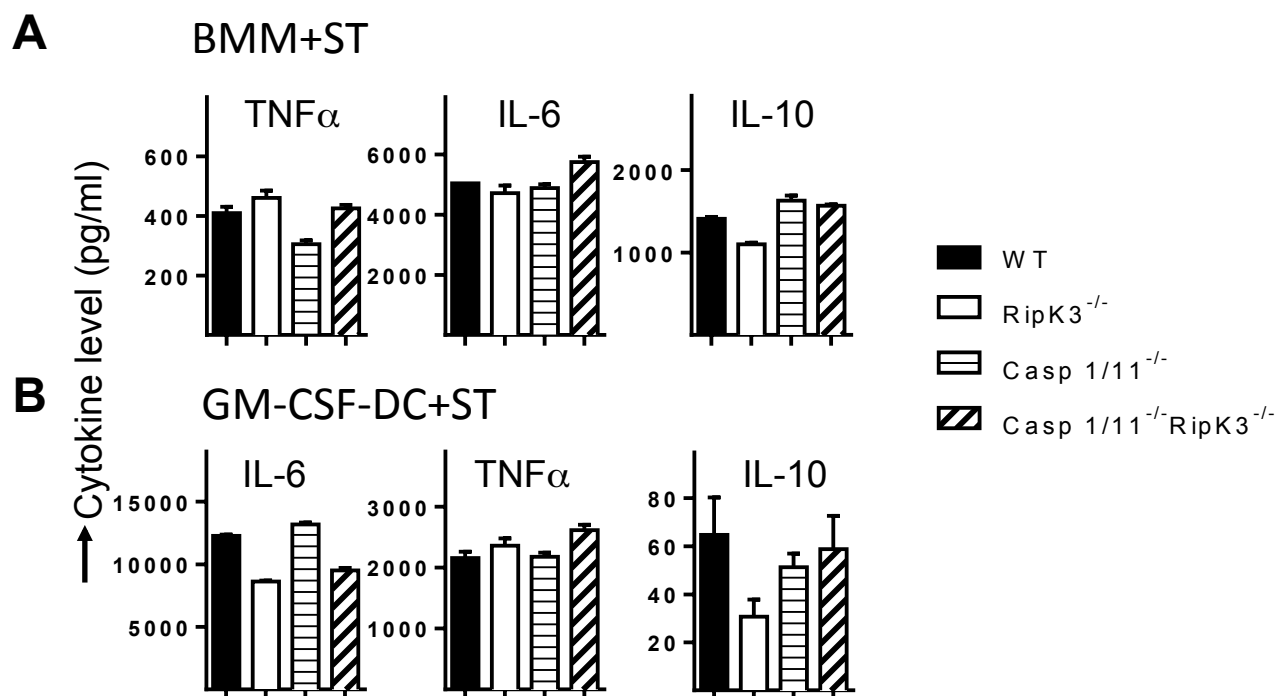


Figure 46: Induction of cytokine production in Caspase1/11- and RipK3-deficient macrophages and DCs following LPS treatment

(A) BMMs and **(B)** GMCSF-DCs were treated with LPS treatment (100ng/ml) for 24 hours and assessed for cytokine production. Data is shown as mean \pm SEM and is representative of 2 separate experiments. All data points represent an n=3 unless otherwise stated.

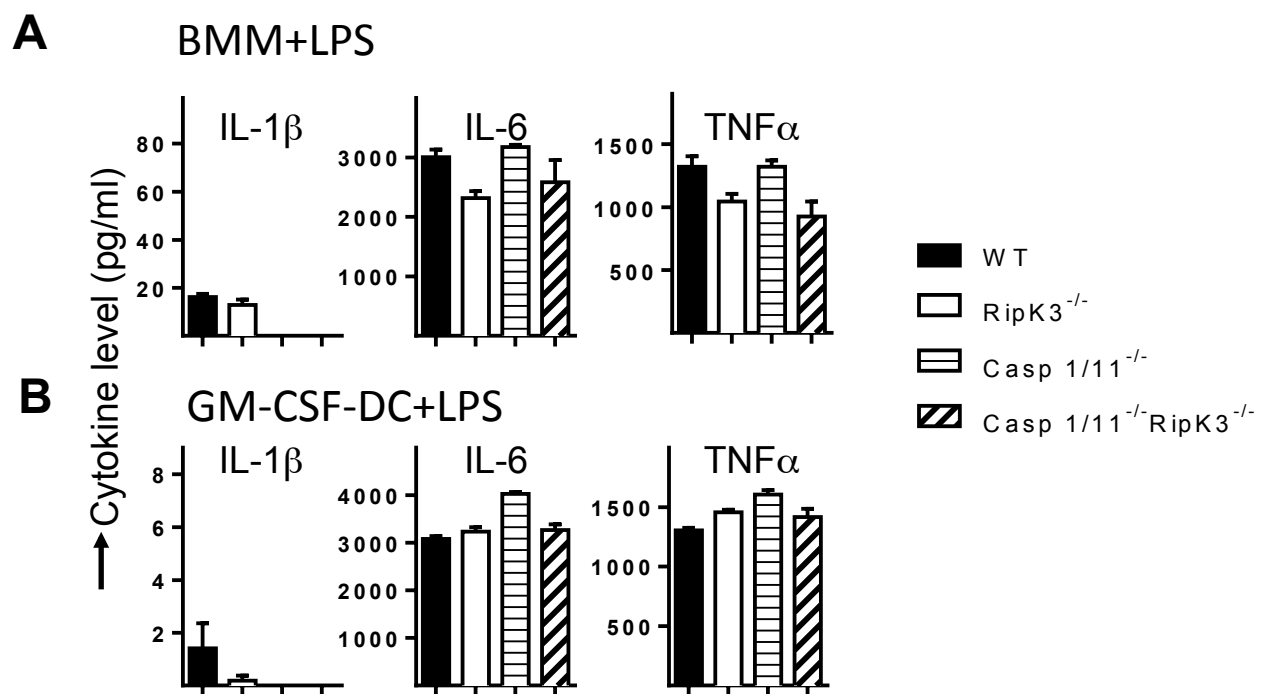


Figure 47: *In-vivo* LPS treatment reveals deficiency in cytokine production in Caspase1/11- and RipK3-deficient mice

WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were injected with LPS *in-vivo* (50 mg/kg LPS *i.p.*). Six hours following administration of LPS, mice were euthanized and serum samples were collected to be assessed for cytokine and chemokine production. Data is shown as mean ±SEM and is representative of 2 separate experiments. All data points represent an n=3 unless otherwise stated.

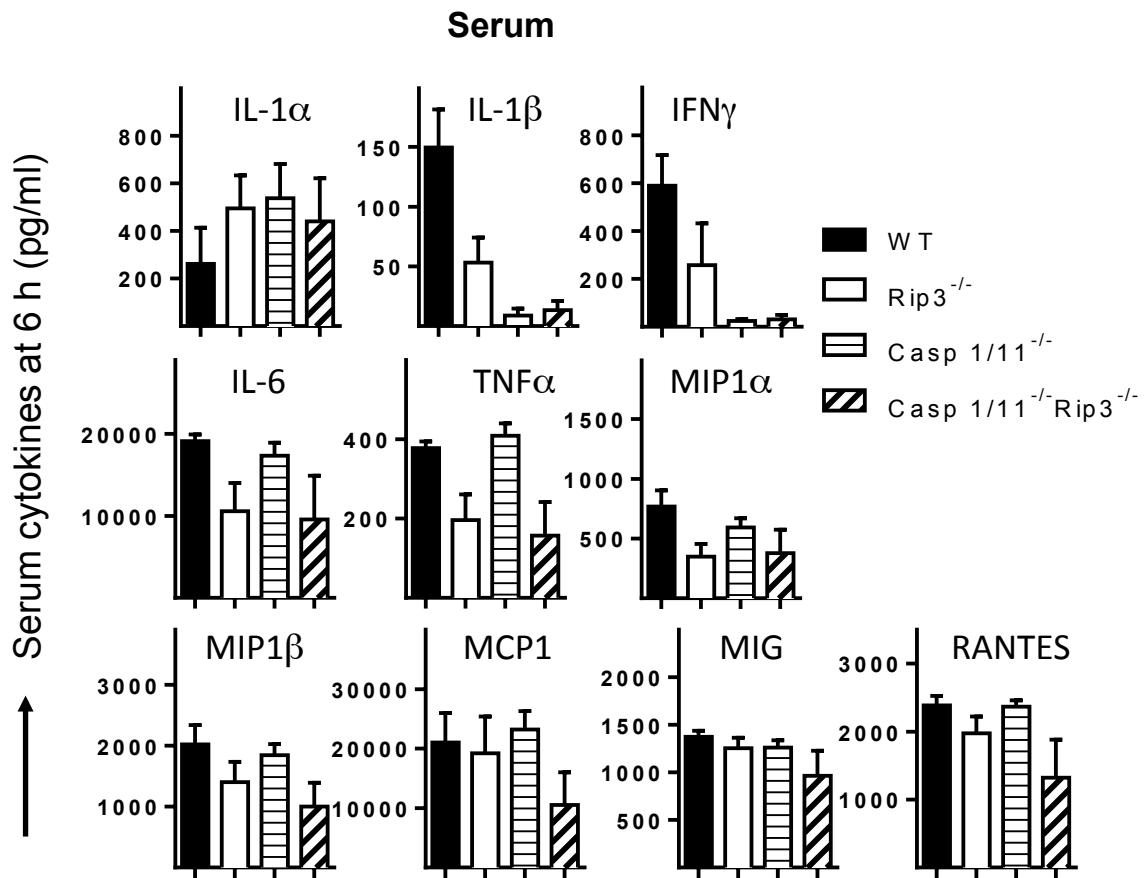


Figure 48: Caspase1/11- and RipK3-deficient mice exhibit attenuation of IL-18 response following ST infection

WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST-OVA-C (10³ CFU *i.v.*). Serum was collected at 5 days post-infection and cytokine production measured by C.B.A assay. Data is shown as mean ±SEM and is representative of 2 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

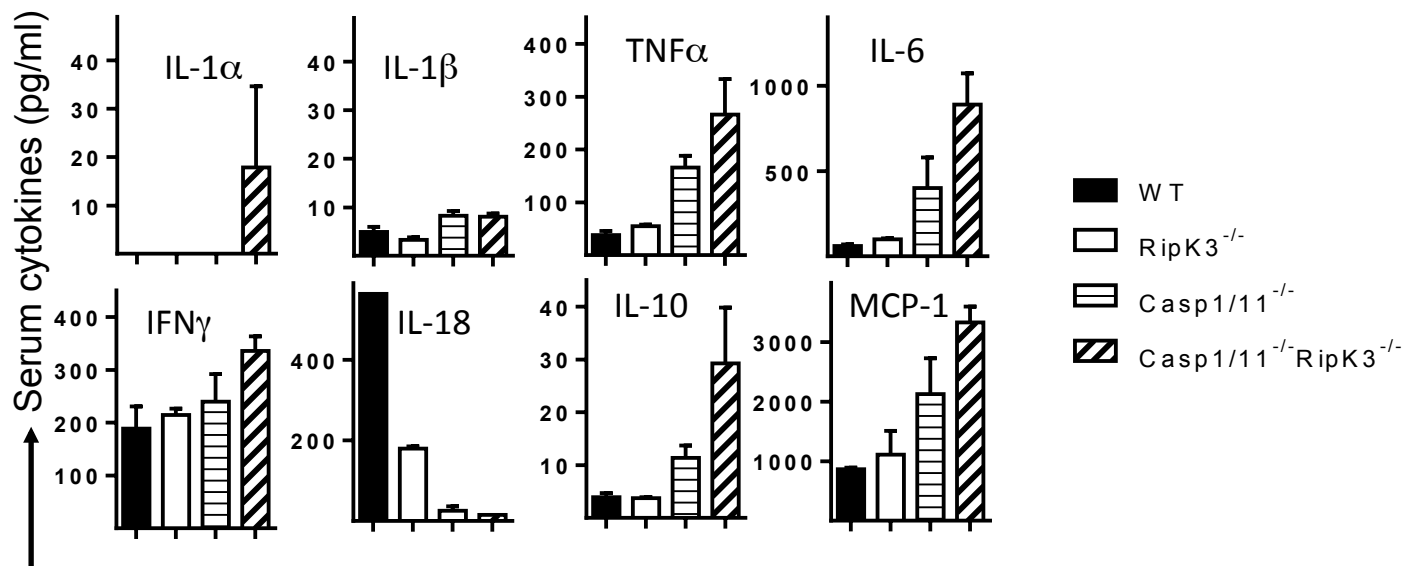
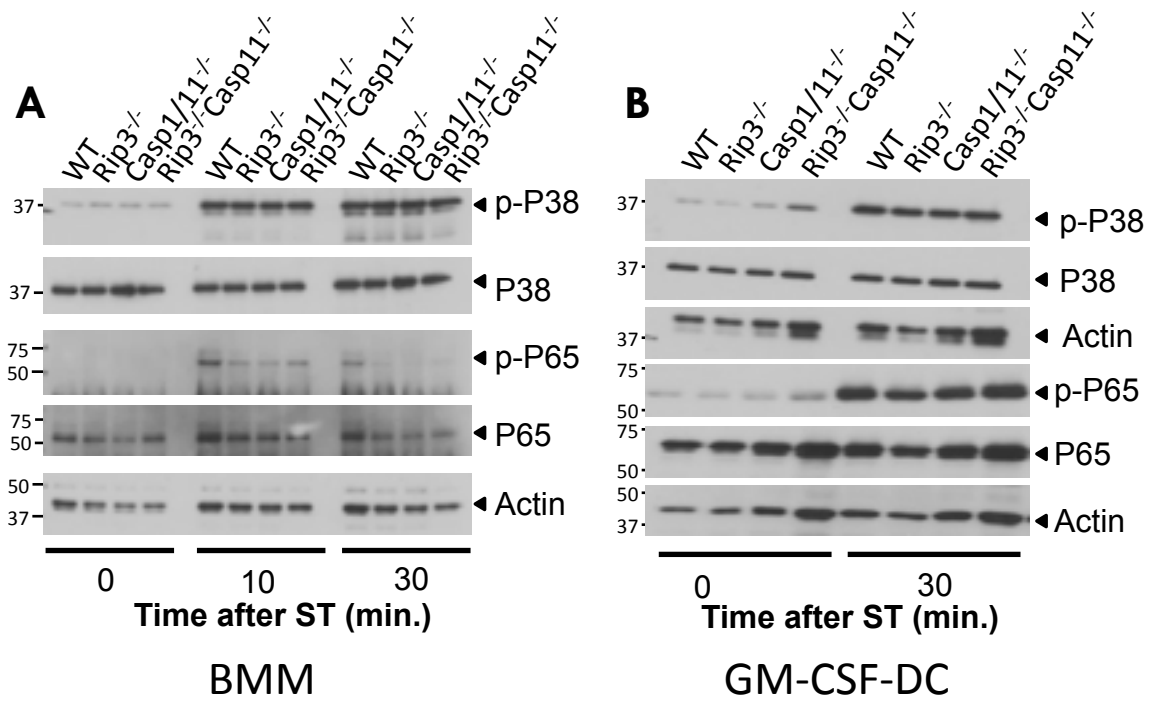


Figure 49: Caspase1/11- and RipK3-deficient macrophages and DCs are able to promote NF- κ B (p65) and MAPK (p38) signalling following ST infection

BMMs (**A**) and GMCSF-DCs (**B**) grown from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST for 30 minutes followed by a 2 hour treatment with gentamycin to eliminate any extracellular bacteria. Cells were infected with ST at an MOI of 10 for 0, 10 and 30 minutes and assessed for NF- κ B (p65) and MAPK (p38) signalling as evaluated in cell lysates by western blot analysis. Each Western blot is a representative of three separate experiments.



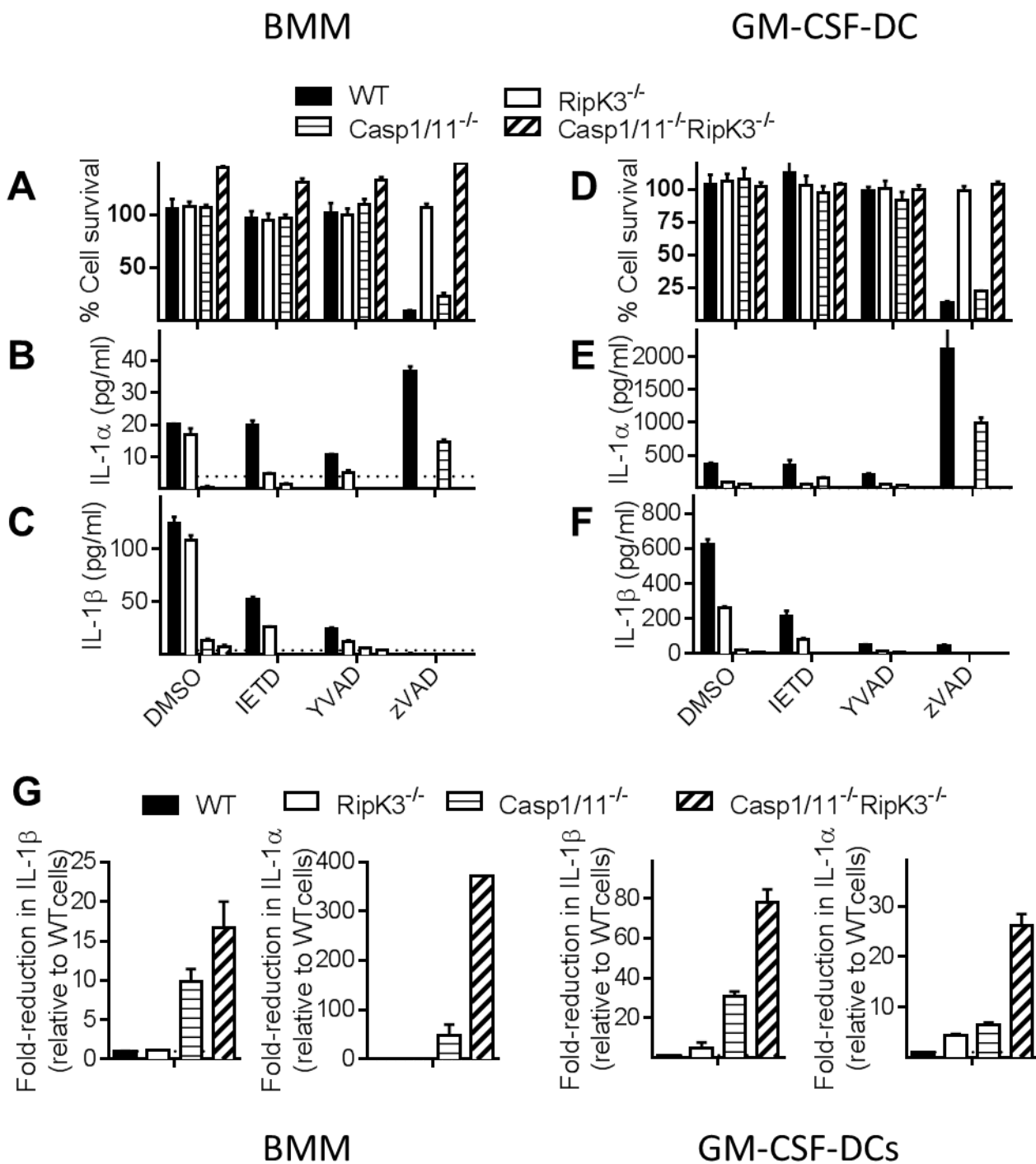
processing of IL-1 cytokines (Signal 2) and not promoting transcription of the pro-forms of IL-1 cytokines (Signal 1).

3.3.5 Caspase-1, Caspase-8 and RipK3 mediate processing of IL-1 cytokines

Processing of IL-1 α and IL-1 β requires two signals to promote secretion (238). The first signal is mediated through PRRs such as TLRs that promote transcription of pro-IL-1 β (385). The second signal is mediated through inflammasome activation, which processes pro-IL-1 β into its active form (385). Pro-IL-1 α is bioactive and is not processed by the inflammasome, instead proteases such as calpains and granzymes can process IL-1 α to increase its pro-inflammatory activity (337). I observed a graded impact of Caspase1/11 and RipK3 on the reduction of IL-1 α and IL-1 β in both BMMs (**Fig. 50A-C**) and GMCSF-DCs (**Fig. 50D-F**). The inhibition of IL-1 α and IL-1 β production in Caspase1/11 and RipK3-deficient cells is further demonstrated by comparing fold reduction of Caspase1/11-RipK3-deficient cells as compared to wild type cells (**Fig. 50G**). Interestingly, Caspase-8 has recently been shown to process pro-IL-1 β into its active form following TLR stimulation (285). To evaluate the impact that Caspase-8 has on Caspase1/11 and RipK3 independent processing of IL-1 cytokines, I utilized chemical inhibitors of Caspase-8 (IETD) and Caspase-1 (YVAD) on BMMs and GMCSF-DCs. I examined the critical role of Caspase-1 and Caspase-8 on the secretion of IL-1 cytokines at low MOIs of ST where cell death was not engaged by BMMs (**Fig. 50A**) or GMCSF-DCs (**Fig. 50D**). Interestingly, even at low MOIs of ST, the pan-Caspase inhibitor (zVAD) was able

Figure 50: Caspase1/11, Caspase-8 and RipK3 are critical signalling pathways to mediate IL-1 β production in macrophages and DCs in response to ST infection

(A-C) BMMs and **(D-F)** GMCSF-DCs were infected with ST at an MOI of 1 and 10 respectively to prevent cell death for 30 minutes followed by 2 hour treatment with gentamycin. Cells were also treated with various inhibitors, including Caspase-8 inhibitor (25 μ M IETD), Caspase-1 inhibitor (25 μ M YVAD) and pan-Caspase inhibitor (25 μ M zVAD). Supernatants were collected following 24 hours of incubation and assessed for cytokine production by CBA assay. Following 24 hours post-infection, **(A)** BMMs and **(D)** GMCSF-DCs were evaluated for viability by neutral red assay. Additionally, supernatants were collected to assess for cytokine production from infected **(B-C)** BMMs and **(D-E)** GMCSF-DCs 24 hours following ST infection. **(G)** Using the data from experiments outlined above, fold reduction of IL-1 cytokines as compared to the wild type group was calculated for IL-1 α and IL-1 β for macrophages and DCs infected with ST. Data is shown as mean \pm SEM and is representative of 2 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



to induce cell death in wild type and Caspase1/11-deficient BMMs and GMCSF-DCs, indicating the engagement of RipK3 dependent necroptosis (**Fig. 50A, D**) since treatment of cells with pan-Caspase inhibitor has been shown to induce necroptosis (386). As expected, cell death induced by zVAD treatment followed excessive release of IL-1 α in the supernatant (**Fig. 50B, E**). Inhibition of Caspase-1 using the chemical inhibitor YVAD greatly reduced the production of IL-1 β by wild type and RipK3-deficient BMMs and GMCSF-DCs, but not in Caspase1/11- or Caspase1/11-RipK3-deficient BMMs and GMCSF-DCs (**Fig. 50C, F**). Inhibition of Caspase-8 by IETD reduced the levels of IL-1 β in all groups tested, with the greatest impact observed in Caspase1/11-RipK3-deficient cells (**Fig. 50C, F**). This suggests that both Caspase-8 and Caspase-1 promote complementary processing of IL-1 β . Furthermore, Caspase-1 mediated processing of IL-1 β is more dominant than that mediated by Caspase-8. When Caspase1/11 and RipK3 pathways were shut down, such as the case with Caspase1/11-RipK3-deficient cells, IL-1 β processing was solely reliant on Caspase-8 processing.

Critical mediators of inflammasome activation such as RipK1, NLRP3, NLRC4, pro-IL-1 β and pro-IL-18 were not reduced in any of the BMMs (**Fig. 51**) and GMCSF-DCs (**Fig. 52**) examined following ST infection or even at naïve state. Interestingly, I observed processing of Caspase-8 in BMMs (**Fig. 51B**) and GMCSF-DCs (**Fig. 52C**) following ST infection only in Caspase1/11-deficient and Caspase1/11-RipK3-deficient cells. This indicates that Caspase-1, the dominant mediator of inflammasome signalling, may be inhibiting the processing of

Figure 51: Macrophages deficient in Caspase1/11 and RipK3 signalling pathways express normal level of proteins critical for inflammation

(A-B) BMMs were infected with ST (at an MOI of 10) for 30 minutes, followed by gentamycin treatment to eliminate any extracellular bacteria. Cell lysates collected from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} BMMs following infection with ST for 0, 2 and 4 hours post infection and assessed by Western blot analysis for the expression of various cytokine precursors and cell death signalling pathways. Each Western blot is a representative of three separate experiments.

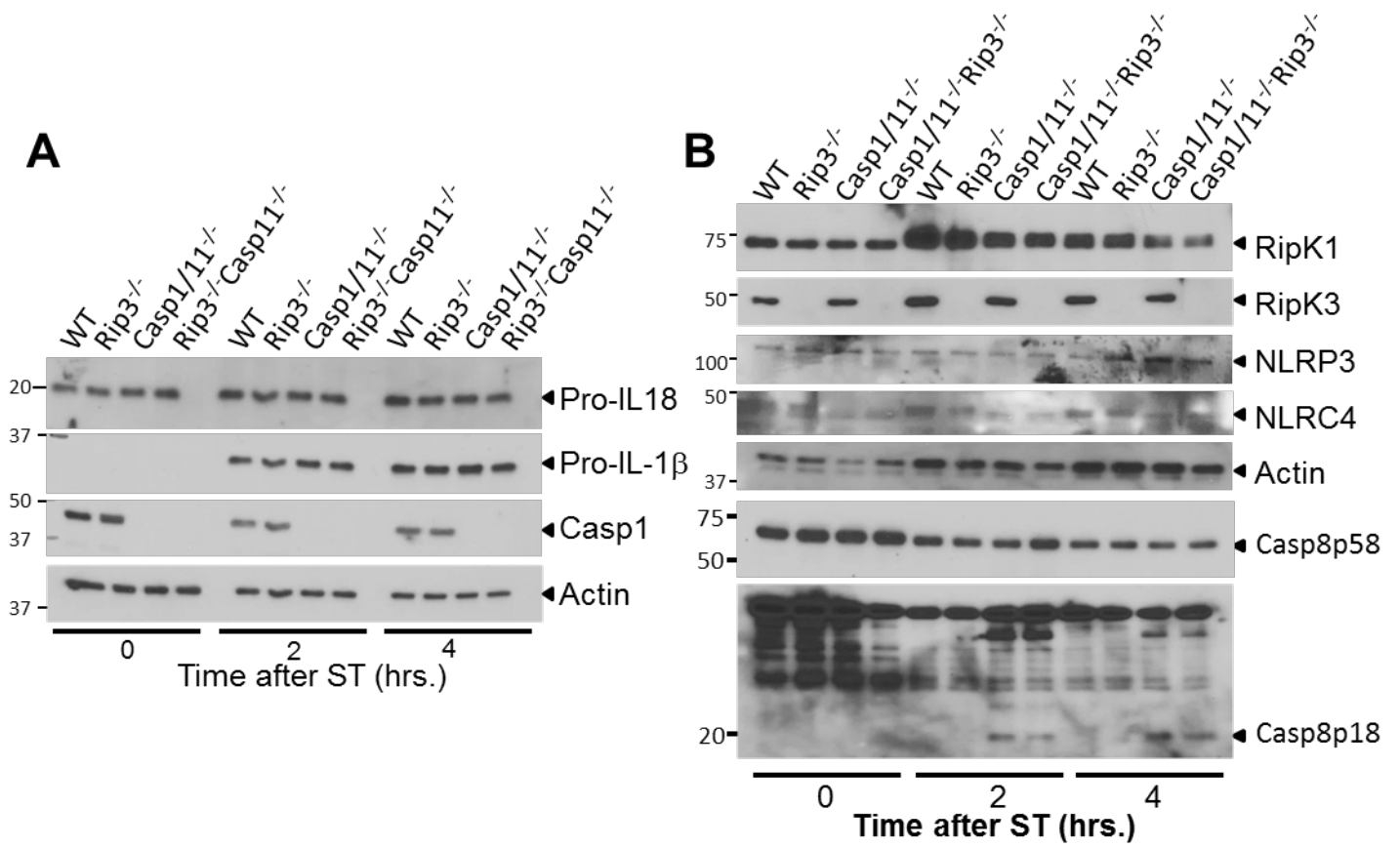
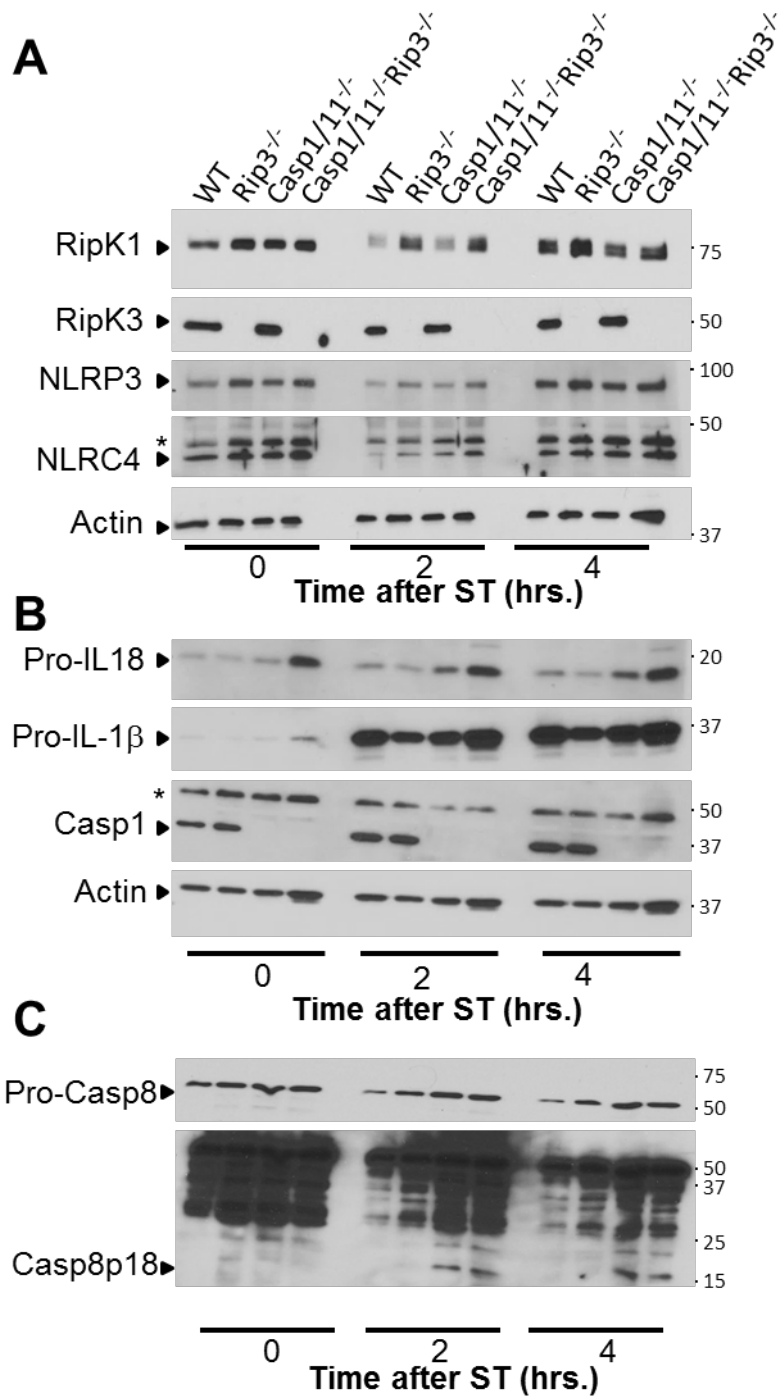


Figure 52: DCs deficient in Caspase1/11 and RipK3 signalling pathways express normal level of proteins critical for inflammation

(A-C) GMCSF-DCs were infected with ST (at an MOI of 10) for 30 minutes, followed by gentamycin treatment to eliminate any extracellular bacteria. Cell lysates collected from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} GMCSF-DCs following infection with ST for 0, 2 and 4 hours post infection and assessed by Western blot analysis for the expression of various cytokine precursors and cell death signalling pathways. Each Western blot is a representative of three separate experiments.



Caspase-8. However, since the Caspase-8 inhibitor IETD was able to reduce IL-1 β levels in all groups tested, I believe wild type and RipK3-deficient cells are able to induce Caspase-8 cleavage which is not detectable in the western blot analysis. In comparison, Caspase1/11 and Caspase1/11-RipK3-deficient cells are producing very large quantities of processed Caspase-8 (p18) as shown by western blot (**Fig. 51, 52**).

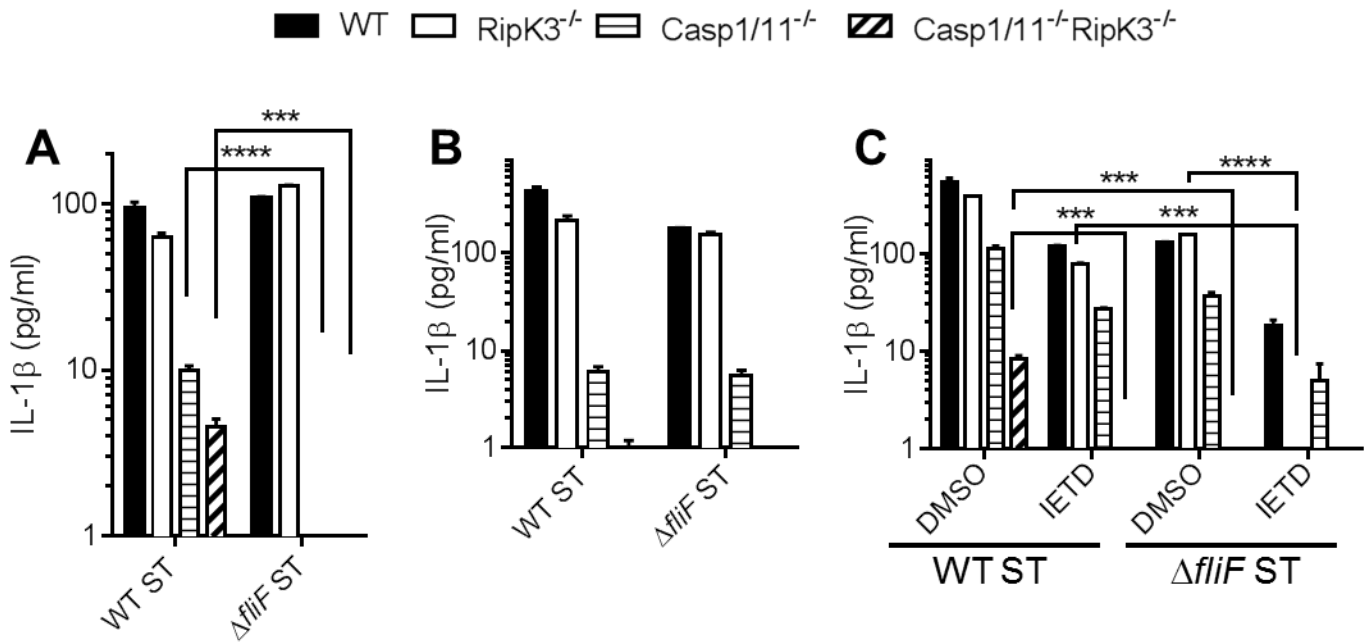
ST expresses many PAMPs such as flagellin that engages TLR signalling and transcription of pro-IL1 β transcripts that are essential for inducing an appropriate inflammatory response (385). Thus I evaluated the inflammatory response mediated by ST Δ FliF (inactivated flagellin) in Caspase1/11- and RipK3-deficient cells. Wild type and RipK3^{-/-} BMMs did not exhibit any attenuation in IL-1 β production following ST Δ FliF infection (**Fig 53A**). In stark contrast, IL-1 β production was greatly reduced following infection of GMCSF-DCs with ST Δ FliF in comparison to wild type ST (**Fig. 53B**). Furthermore, the greatest reduction observed was in the Caspase1/11-RipK3-deficient GMCSF-DCs, indicating that the double knockout cells are highly reliant on flagellin detection for induction of IL-1 β production (**Fig. 53B**). Treatment with IETD revealed that inhibition of Caspase-8 yielded a greater reduction in IL-1 β production by RipK3-deficient GMCSF-DCs following ST Δ FliF infection (**Fig. 53C**).

3.3.6 Regulation of inflammasome activation by RipK3 kinase activity

RipK3 can mediate regulation of multiple signalling pathways by either its kinase activity or its scaffolding properties (269). For instance, RipK3-deficient

Figure 53: In the absence of Caspase1/11, Caspase-8 and RipK3 are critical signalling pathways to mediate IL-1 β production against flagellin in DCs

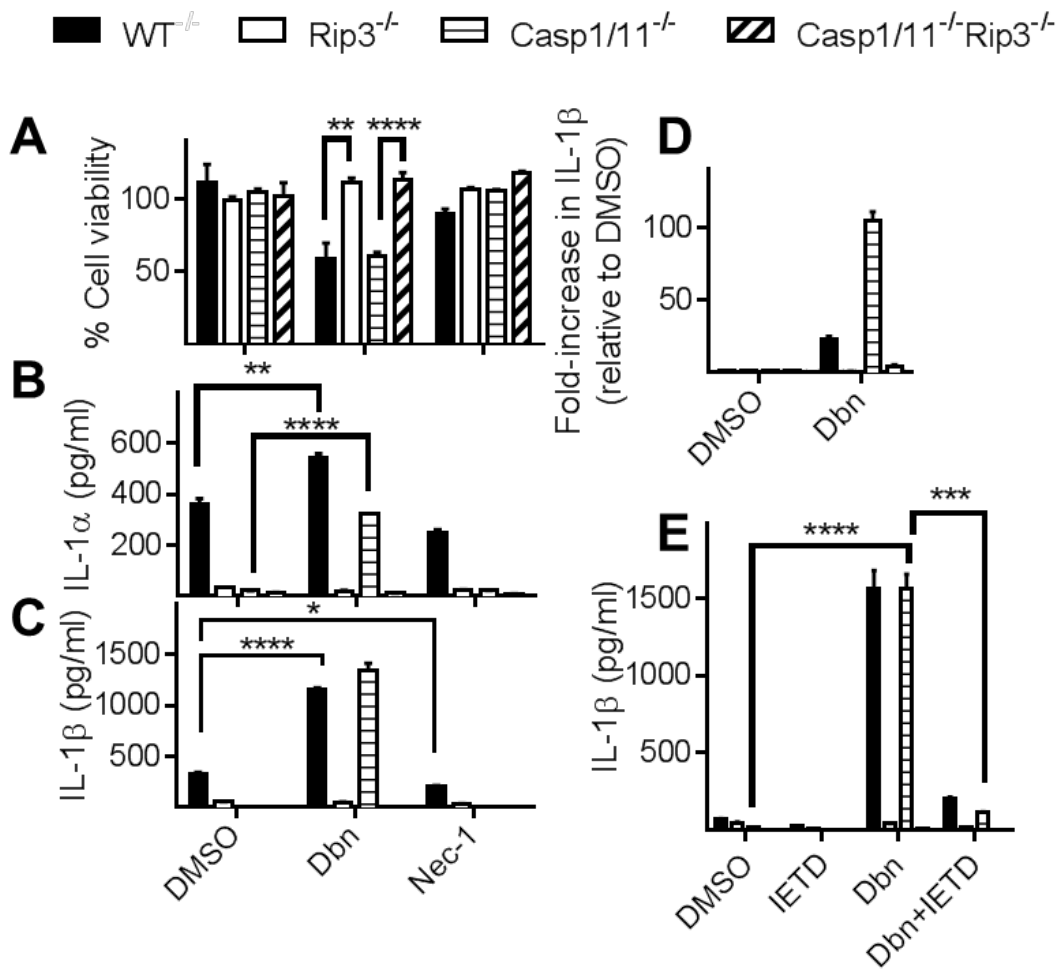
(A) BMMs and **(B)** GMCSF-DCs were infected for 30 minutes with ST and ST Δ *fliF*, a mutant of ST that lacks expression of flagellin. To limit cell death, BMMs were infected at an MOI of 1 and GMCSF-DCs were infected with an MOI of 10. Following a 2 hour gentamycin wash and 24 hour incubation, supernatants were collected and analyzed for IL-1 β production. **(C)** GMCSF-DCs were also infected with ST and ST Δ *fliF* in the presence or absence of Caspase-8 inhibitor (25mM IETD). The production of IL-1 β was measured in the supernatant following 24 h incubation. (Data is shown as mean \pm SEM and is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (**p<0.001, ****p<0.0001).



mice are viable, however deletion of the kinase region of RipK3 results in embryonic lethality, due to overt activation of apoptotic cell death (315). I utilized Dabrafenib (Dbn), an inhibitor of RipK3 kinase activity which selectively binds to the ATP-binding pocket of RipK3 (387), to determine whether the scaffolding property or the kinase activity of RipK3 was regulating IL-1 cytokine production. Surprisingly, treatment of GMCSF-DCs with Dbn during infection with low MOIs of ST resulted in high levels of cell death only in wild type and Caspase1/11-deficient GMCSF-DCs, while RipK3- and RipK3-Caspase1/11-deficient GMCSF-DCs were unaffected (**Fig. 54A**). Consequently, Dbn promoted excessive processing of IL-1 α and IL-1 β in wild type and Caspase1/11-deficient DCs, indicating that the kinase region of RipK3 plays an inhibitory role in IL-1 β production, independent of the processing activity of Caspase1/11 (**Fig. 54B-D**). Furthermore, the massive induction of IL-1 β by Dbn treatment was dependent on Caspase-8 activation, as co-treatment with IETD greatly diminished these levels (**Fig. 54E**). In comparison, the kinase activity of RipK1 plays a modest, but important role in IL-1 β production as GMCSF-DCs treated with Nec1 (RipK1 kinase inhibitor) induced lower levels of IL-1 β in all groups tested. This data indicates a dual functionality of RipK3. Accordingly, its scaffolding properties promote inflammasome activation while its kinase region inhibits it. Additionally, inhibiting RipK3 kinase activity induces potent inflammasome activation that is dependent on RipK3's scaffolding properties and Caspase-8 dependent pathways.

Figure 54: Inhibition of RipK3 kinase activity potentiates IL-1 β cytokine production in DCs

GMCSF-DCs from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST at 1 MOI for 30 minutes and treated with with RipK3 inhibitor Dabrafenib (25 μ M, Dbn), RipK1 inhibitor Necrostatin (25 μ M, Nec-1) and Caspase-8 inhibitor (25 μ M, IETD). Cells were then treated with gentamycin for 2 hours, followed by a 24 hour incubation. Inhibitor concentrations were maintained throughout the entire experiment subsequent to each wash. **(A)** Cells were assessed for viability using the neutral red assay. Additionally, supernatants were collected 24 hours post infection and assessed for **(B)** IL-1 α and **(C-E)** IL-1 β cytokine production. Data shown is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



3.3.7 Regulation of inflammasome activation by cellular inhibitors of apoptosis proteins

Cellular inhibitors of apoptosis (cIAPs) are critical regulators of apoptotic cell death and have also been shown to impact inflammation following various stimuli (222). However, their role in regulating IL-1 β production remains controversial (388, 389). Thus, I tested the role of cIAPs in inflammasome activation to determine how cIAP can regulate RipK3 and Caspase-1/11 signalling pathways. I infected GM-CSF-DCs at low MOI of ST, which does not normally induce cell death. Interestingly, treatment with SMAC mimetic (inhibitor of cIAP1/2) at these low levels of ST infection was able to induce death in all groups tested, albeit to a lesser degree in RipK3-deficient GM-CSF-DCs (**Fig. 55A**). Treatment of SMAC mimetic also correlated with increased production of IL-1 α and IL-1 β (**Fig. 55B, C**). This indicates that cIAPs negatively regulate Caspase1/11 and RipK3 dependent inflammasome pathways. Furthermore, regulation of IL-1 β production mediated by cIAPs was most evident in Caspase1/11-Rip3-deficient GM-CSF-DCs (**Fig. 55D**). This data indicates that cIAPs mediate dual regulation of Caspase1/11 and RipK3 pathways of IL-1 β secretion (**Fig. 55D**). Furthermore, inhibition of cIAPs can also promote IL-1 β processing through Caspase-8 activation, as treatment with IETD greatly diminishes IL-1 β levels induced by cIAP inhibition (**Fig. 55E**). Interestingly, cIAP1/2 protein levels were augmented in Caspase1/11-deficient GM-CSF-DCs (**Fig. 56A, B**) and BMMs (**Fig. 56B**). This suggests that Caspase1/11 down-regulates cIAP levels. Thus, ablation of

Figure 55: cIAP1/2 negatively regulates IL-1 β cytokine production in DCs

GMCSF-DCs from WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice were infected with ST at an 1 MOI for 30 minutes, incubated with SMAC mimetic, Birinapant (5 μ M, Bir) and (E) Caspase-8 inhibitor (25 μ M, IETD). Cells were then treated with gentamycin for 2 hours, followed by a 24 hour incubation. Inhibitor concentrations were maintained throughout the entire experiment subsequent to each wash. (A) Cells were assessed for viability using the neutral red assay. Additionally, supernatants were collected 24 hours post infection and assessed for (B) IL-1 α and (C-E) IL-1 β cytokine production. Data shown is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (**p<0.01, ***p<0.001).

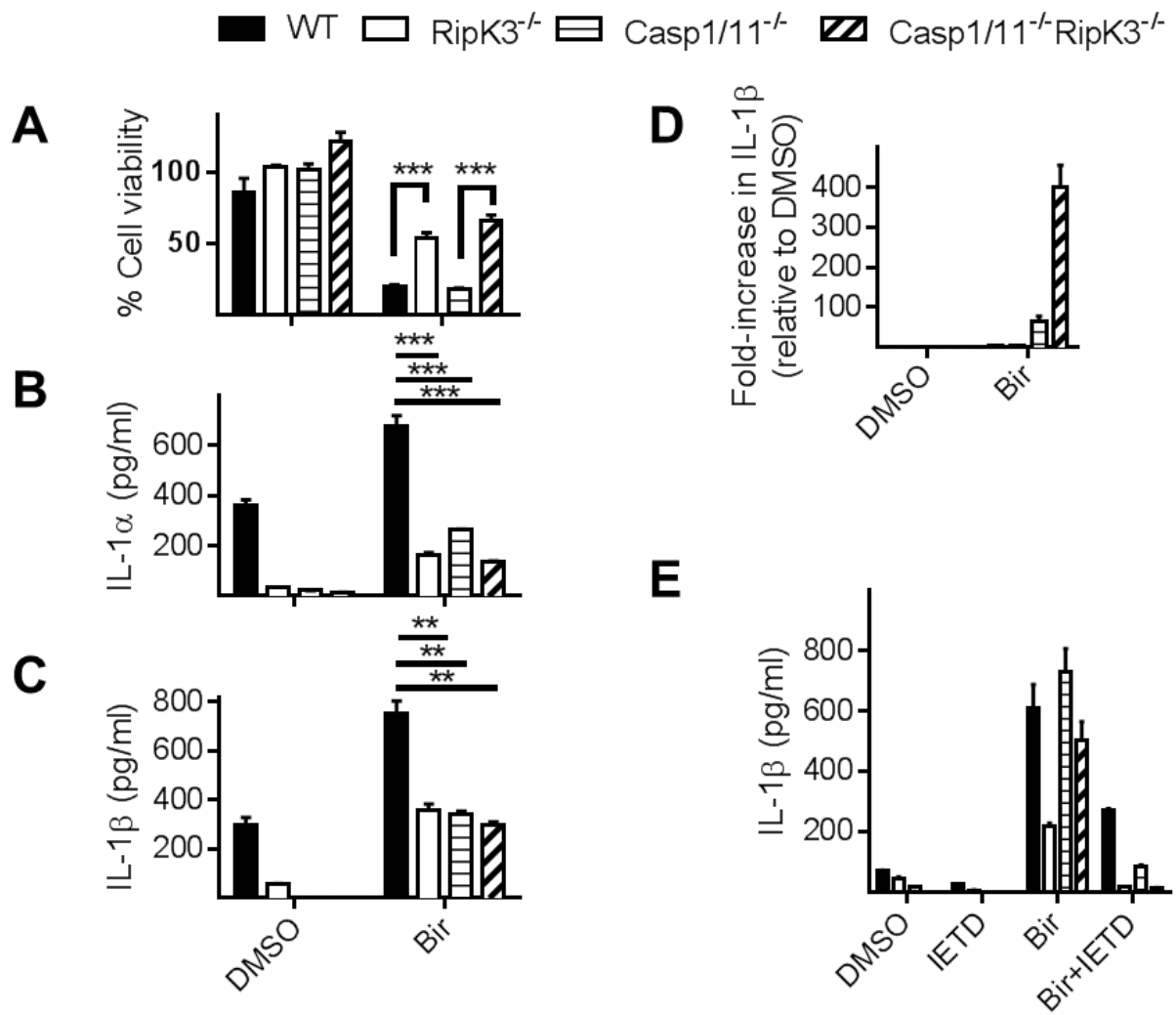
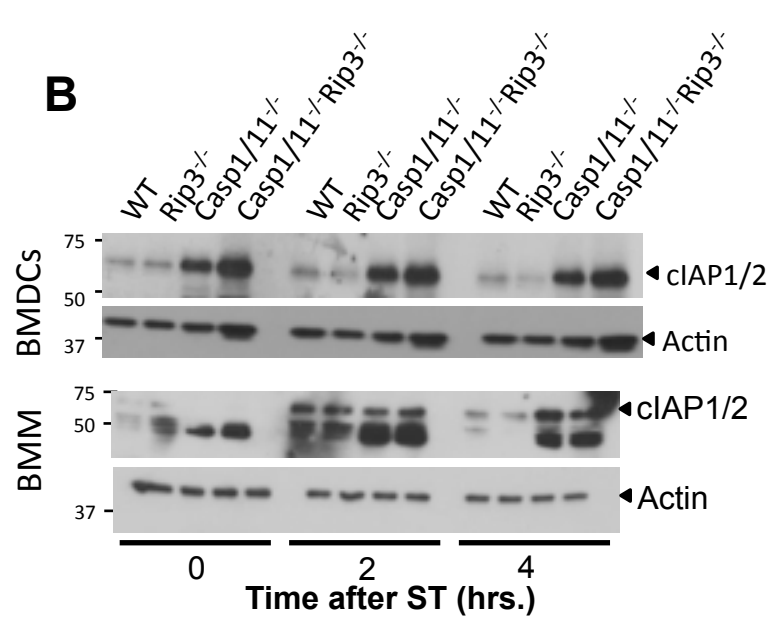
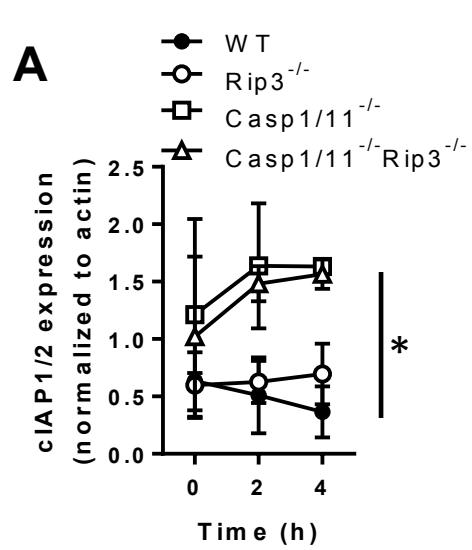


Figure 56: In the absence of Caspase1/11, cIAP1/2 expression is increased

Western blot analysis was performed on cell lysates collected following infection of WT, RipK3^{-/-}, Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} GMCSF-DCs with ST at an MOI of 10) at 0, 2 and 4 hours following infection. **(A)** Densitometry analysis of cIAP1/2 expression following ST infection was assessed for GMCSF-DCs. **(B)** Representative Western blot analysis is shown for cIAP1/2 expression in GMCSF-DCs and BMMs following ST infection. Data shown is representative of 3 separate experiments. All data points represent an n=3 unless otherwise stated. Each Western blot is a representative of three separate experiments. Statistical significance was determined by unpaired student t-test (*p<0.05).



Caspase1/11 results in increased levels of cIAP1/2. These results suggest that Caspase1/11 modulates both Caspase-8 processing and cIAP expression levels, which maintains the dominance of the Caspase-1 pathway to promote inflammasome activation.

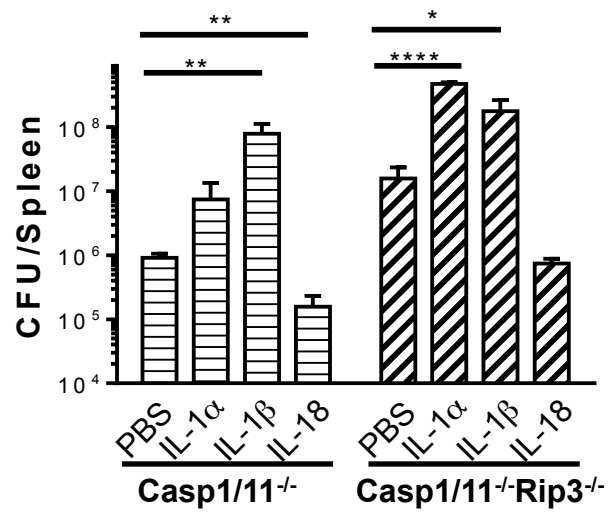
3.3.8 Administration of IL-18 reduces the ST bacterial burden in Caspase1/11 and RipK3-deficient mice

My data indicates that RipK3, Caspase1/11 and Caspase-8 promote the processing of IL-1. Furthermore, Caspase1/11-RipK3-deficient mice rapidly succumb to infection with recombinants of ST that induces minimal lethality in wild type mice (**Fig. 42B**). I also observe increased mortality in Caspase1/11- and to a lesser extent in RipK3-deficient mice (**Fig. 42B**). To test the impact of reduced levels of IL-1 cytokines on ST infection, I injected recombinant murine IL-1 β and IL-18 in Caspase1/11- and Caspase1/11-RipK3-deficient mice following infection with ST (ST-OVA-C) (**Fig. 57**). Administration of exogenous IL-18 reduced the bacterial burden in both Caspase1/11- and Caspase1/11-RipK3-deficient mice. In stark contrast, administration of exogenous IL-1 β exacerbated ST infection (**Fig. 57**). This result is most likely due to the fact that IL-1 β can act as a DAMP, which can induce overt inflammation and immune pathology.

My results indicate that there are several checkpoints that regulate inflammasome activation. While RipK3 promoted Caspase-8-dependent inflammasome activation, this interaction was regulated by the kinase region of RipK3. There are conflicting reports regarding activation (388) or regulation (389) of Caspase-1 by cIAPs. My results indicate that cIAPs regulate inflammasome

Figure 57: IL-18 plays a protective role in Caspase1/11- and RipK3-deficient mice following ST infection

Mouse recombinant protein IL-1 α (100 ng/mouse, *i.p.*), IL-1 β (100 ng/mouse, *i.p.*) and IL-18 (1000 ng/mouse, *i.p.*) was injected into Caspase1/11^{-/-} and Caspase1/11^{-/-}RipK3^{-/-} mice daily from day -1 to 2 post-infection with ST-OVA-C (10³ CFU, *i.v.*). Spleens were harvested from infected mice on day 6 following infection and the bacterial burden was evaluated for each mouse group. Data shown is representative of 2-3 separate experiments. All data points represent an n=3 unless otherwise stated. Statistical significance was determined by unpaired student t-test (*p<0.05, **p<0.01, ****p<0.0001).



activation at various steps (**Fig. 58**), and interestingly, Caspase-1 appears to regulate the expression of cIAPs and Caspase-8-processing, thereby explaining why Caspase-1 plays a dominant role in inflammasome activation (**Fig. 58**). Overall, I have revealed the various pathways through which Caspase-1, Caspase-8 and RipK3 participate in inflammasome activation, and how it impacts host survival against an infection with a virulent intracellular bacterium (**Fig. 59A-C**).

Figure 58: Visual Abstract III-A: Caspase1/11, Caspase-8 and RipK3 promote comprehensive inflammasome activation against ST

Protective immune defenses against ST can be mediated by inflammasome activation and processing of inflammatory cytokines pro-IL-1 β and pro-IL-18 into their active forms. Four distinct pathways can engage inflammasome activation against ST: **(1)** a Caspase1-Caspase-8 co-dependent pathway, **(2)** a Caspase-1 dependent pathway, **(3)** a Caspase8-RipK3 dependent pathway, **(4)** a Caspase-8 dependent pathway, which requires detection of flagellin. Interestingly, RipK3 promotes inflammasome activation independent of its kinase activity. Furthermore, cIAP1/2 and RipK3 kinase activity can promote or inhibit inflammasome activation by regulating Caspase-1 and Caspase-8 activity. Caspase-1 can regulate the expression of cIAP1/2, which then prevents processing of Caspase-8. The kinase activity of RipK3 can negatively regulate activation of Caspase-8. When RipK3 kinase inhibitors are used in the context of an ST infection, we observe optimal inflammasome activation independent of Caspase1/11. Comprehensively, Caspase1/11, Caspase-8 and RipK3 promotes optimal inflammasome activation against ST, which is regulated by cIAP1/2 and RipK3 kinase activity.

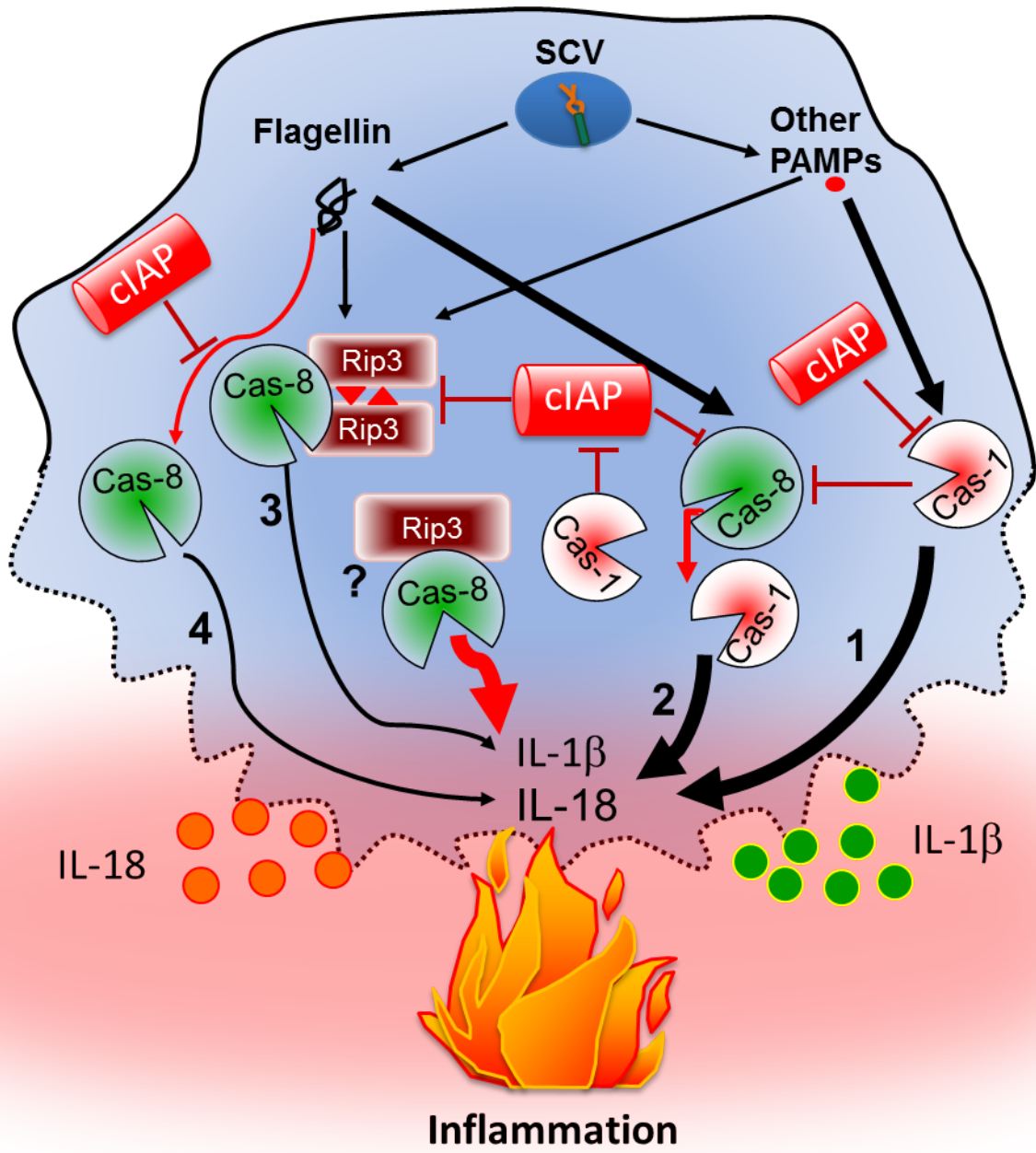
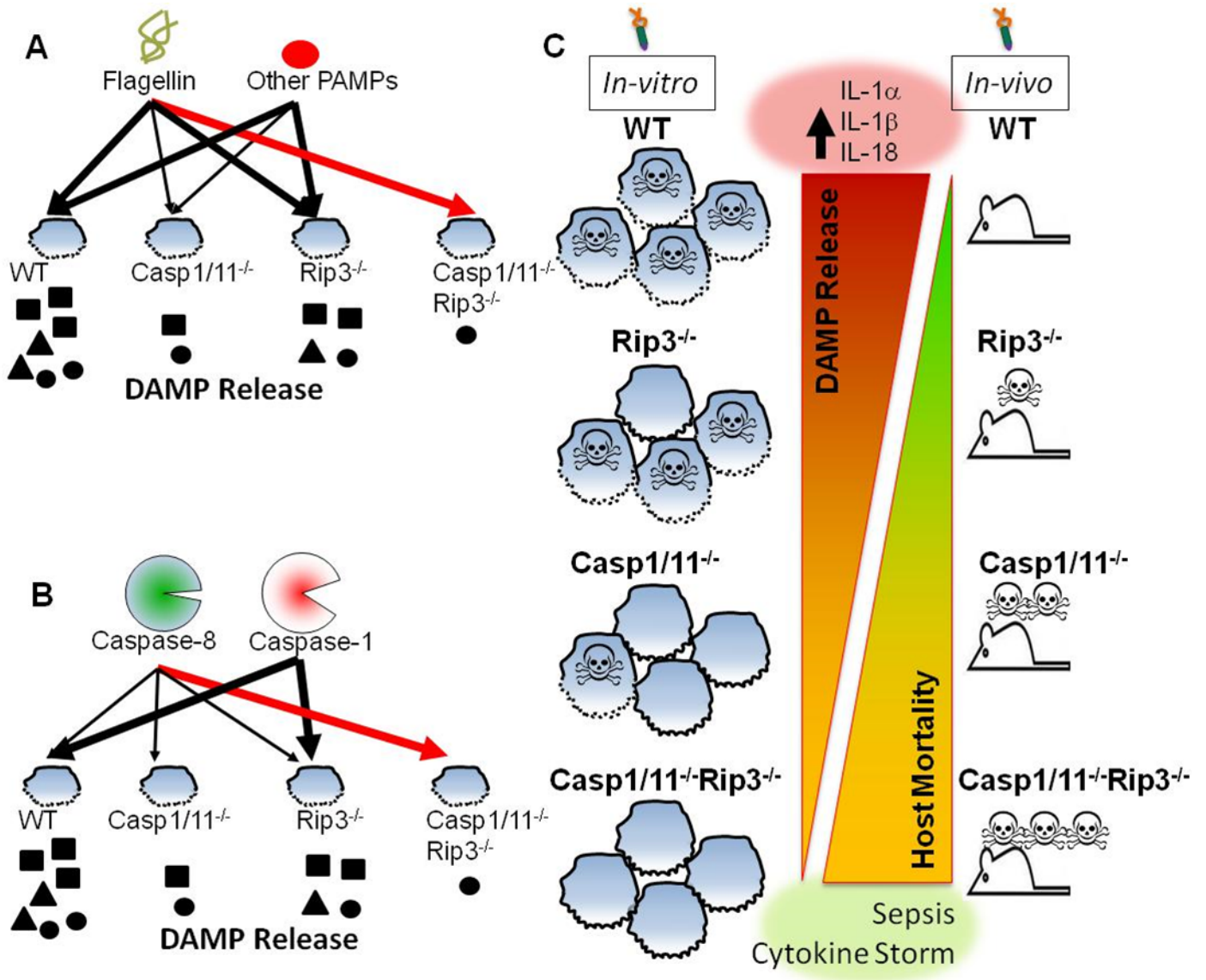


Figure 59: Visual Abstract III-B: Impact of Caspases and RipK3 on inflammasome activation and host survival against ST

(A) Flagellin and other bacterial PAMPs are able to induce inflammasome activation through Caspase-1, Caspase-8 and RipK3-dependent signalling pathways. Flagellin mediated inflammasome activation against ST becomes a critical pathway when both Caspase1/11 and RipK3 signalling platforms are shut down. **(B)** Caspase-1 is a dominant pathway for mediating inflammasome activation following ST infection. Caspase-8 represents an additional pathway to engage inflammasome activation against which is critical only when Caspase-1 and RipK3 is absent. **(C)** Both RipK3 and Caspase1/11 signalling pathways promote inflammation and subsequent DAMP release. Interestingly, engagement of cell death pathways and inflammation promotes host survival against virulent ST, as our data suggests a graded exacerbation in bacterial burden following infection of mice with different recombinants of ST (Caspase1/11^{-/-}RipK3^{-/-} > Caspase1/11^{-/-} > RipK3^{-/-} > WT).



4: DISCUSSION

DCs provide a critical link between the innate and adaptive immune response to promote a comprehensive control of a variety of pathogens by limiting pathogenic burden and inducing an acquired immune response that provides sustained surveillance against pathogens (2, 51). Initially, their *in-vivo* relevance and cellular autonomy from the closely related macrophage population was under intense debate (50, 137, 390). However, seminal studies following the initial study of Ralph Steinman and Zanvil Cohn have utilized a variety of approaches to conclusively demonstrate the critical impact of DCs on mediating immunity (125, 137, 358, 391-395). Various subsets of DCs have been identified that promote immune protection against a variety of pathogens (52). My studies show that CD8 α DCs and CD103⁺ DCs play an important role in inducing a CD8⁺ T cell response against ST (**Fig 28-31, 34**), which is required for effective clearance of virulent ST. Furthermore, my studies indicate that IFN-I signalling in APCs is critical in mounting a robust CD8⁺ T cell response against ST (**Fig. 18**). Indeed, IFN-I signalling promotes secretion of inflammatory cytokines and chemokines in DCs infected with ST (**Fig. 23, 24**). In addition, ST mediated cell

death was diminished in IFNAR1-deficient DCs that are dependent on Batf3 (FLT3L-DCs and CD103i DCs) (**Fig. 22**). I also observed enhanced recruitment of immune cells to the draining lymph nodes of ST infected mice, which correlated with a robust CD8⁺ T cell response (**Fig. 18, 25-26**).

In addition, my studies reveal that DCs mediate immune protection against ST by engaging appropriate cell death pathways to limit dissemination of ST and promote host survival (**Fig 38-41**). Cell death pathways mediated by RipK3 and Caspase1/11 can promote inflammation through the release of various DAMPs such as ATP, uric acid, and HMGB1, which is limited following induction of apoptosis and sequestered in healthy cells (216). Furthermore, RipK3 and Caspase1/11 can also promote processing of IL-1 inflammatory cytokines such as proIL-1 β and pro-IL-18 into their bioactive forms (237, 396, 397). Furthermore, by engaging cell death, DCs can mediate antigen availability to promote cross-presentation of cellular antigen by neighbouring DC to induce an adaptive CD8⁺ T cell response (113, 220, 382). Pathogen can utilize an array of virulence factors to subvert engagement of appropriate cell death pathways to limit inflammation, antigen availability and antigen presentation to limit both the innate and adaptive arms of the immune response (398).

Following infection with ST, susceptible inbred mouse strains (B6, Balb/c) rapidly succumb to infection, while resistant mice (129, CBA, C3H) maintain a chronic infection that lasts for months (161, 179). For instance, ST induces rapid host fatality in B6 mice (<7 days) (161). Although many genetic differences are present between resistant and susceptible mice strains, one key determinant for

host survival against ST has been identified as *Nramp1* (179). Susceptible mice express a mutation in the *Nramp1*^{D169} gene, resulting in a missense mutation on the fourth transmembrane resulting in a non-functional protein (179). In contrast, resistant mice express a functional *Nramp*^{G169} gene, limiting pathogen replication in the phagosome and subsequent elimination of phagosomal pathogens, such as Mycobacteria, Leishmania and Salmonella (179). B6 mice are the general background of choice for many relevant knockout models. However, this makes it difficult to study the impact of various genetic deficiencies during ST infection due to premature death of B6 mice. In order to assess multiple facets of the innate and adaptive immune responses against ST, I utilized the B6 mouse model that expresses a functional *Nramp*^{G169} gene (171). Interestingly, transgenic expression of functional Nramp1 protein extends the survival of susceptible mice, albeit only by 2-3 weeks (**Fig. 10B**). In comparison, F3.B6129 mice expressing functional *Nramp*^{D169/D169} maintain a chronic infection that lasts for months (**Fig. 9B**). This data suggest that resistant strain of mice express additional genes that promote protection against ST. Indeed, other resistance genes have been associated with protection against ST (399). Nevertheless, utilizing the B6.Nramp mouse model allowed us an additional time frame to assess the impact of adaptive immune response against ST to decipher critical components of the immune response against ST that are still under debate.

It is critical that both the innate and adaptive immune responses work in concert to mediate immune protection against ST (364, 400). Interestingly, both CD4⁺ and CD8⁺ T cell responses are greatly delayed following ST infection (161,

401, 402). Furthermore, neutralization of IFN- γ has been shown to result in resurgence of ST during later stages (370). The protective role of CD4⁺ T cells during infection with attenuated strains of ST has previously been characterized (366, 402, 403). In comparison, the role of CD8⁺ T cells against attenuated ST has been somewhat controversial and has been shown to be secondary to CD4⁺ T cells (365, 369, 403). However the role of CD8⁺ T cells against primary infection with virulent ST remains to be elucidated. This is partly due to the necessity of using auxotrophic or attenuated strains of ST to extrapolate the role of CD8⁺ T cells during ST infection as pathogenic strains induce rapid lethality in relevant mouse models (bred onto susceptible mouse strains) (349, 366, 369). Indeed CD8⁺ T cells play a key protective role during infection of mice with other virulent intracellular bacteria such as *M. tuberculosis*, as MHC class-I-deficient mice become moribund within the first month of infection (348). Other groups have depleted CD8⁺ T cells using anti-CD8 antibodies (404) or used β 2m-deficient mice lacking MHC class-I (349), to assess the importance of CD8⁺ T cells against avirulent strains of ST. These studies indicated that CD8⁺ T cells play a limited role during infection with ST.

My study indicates that both CD4⁺ and CD8⁺ T cells play an important role during primary infection with virulent ST. Pathogen virulence is expected to induce significant pathology, and all the facets of the immune response must therefore be engaged for host survival. My study found that CD8⁺ T cells mediate protection against virulent ST in both the susceptible (B6.Nramp^{G169}) and resistant (129) strains of mice. Use of the B6.Nramp mouse model allowed us to

evaluate the critical role of CD8⁺ T cells against virulent ST infection (**Fig. 10, 41**). In the absence of any CD8⁺ T cells, mice succumb faster to systemic ST infection in comparison to wild type controls (**Fig. 9C, 10C**). Furthermore, antibody mediated depletion of CD8⁺ cells resulted in higher bacterial burdens as compared to isotype controls (**Fig. 9CD, E**). Continuous surveillance by T cells against chronic pathogens and resurgence of the pathogen owing to immune suppression during later stages of life seems to be a common paradigm during infection with phagosomal pathogens (405). I have also observed that the CD8⁺ T cell response against ST is dependent on CD4⁺ T cells (**data not shown**), so it is likely that in case of CD4⁺ T cell depletion, CD8⁺ T cell response is also compromised. These observations need to be kept in perspective when interpreting the results of CD4⁺ versus CD8⁺ cell depletions.

ST is an intracellular bacterium that is capable of sequestering itself within the phagosome of infected cells. Normally, the phagosome maintains a harsh environment to facilitate pathogen destruction, allowing sentinel cells, such as macrophages and DCs, to eliminate pathogens following phagocytosis. ST utilizes virulence genes encoded in SPI genes to induce pathogen uptake and create a niche in the phagosomal environment (166). Infection of mice with ST results in activation of CD8⁺ T cells as revealed by the increase in the numbers of CD44^{hi}, CD62L^{low} cells by flow cytometry (160). Further characterization of specific CD8⁺ T cell responses are challenging, as the endogenous epitopes of ST that are presented by APCs to CD8⁺ T cells are not known. Thus recombinants of ST have been used that express foreign proteins, such as OVA,

to measure the response against ST. I utilized two virulent recombinants of ST expressing the model antigen OVA that is either expressed in the phagosome (ST-OVA) or translocated to the cytoplasm (ST-OVA-C) of infected cells (**Fig. 16, 17**). My results indicate that CD8⁺ T cell responses against ST are critical for immune protection against the bacterium irrespective of the location of antigen (**Fig. 9-11, 28-31**) (369). ST-OVA maintain all antigens in the phagosome (46). Thus CD8⁺ T cell priming requires cross-presentation of phagosomal antigens for antigen presentation to CD8⁺ T cells, which perceived to be delayed during ST infection (46, 161). In contrast, priming of CD8⁺ T cells against ST-OVA-C is more rapid as OVA antigen is deposited in the cytoplasm of infected cells, which can engage direct proteosomal antigen processing and allows for robust activation of CD8⁺ T cells (46). Interestingly, infection with ST-OVA-C results in a rapid CD8⁺ T cell response, and survival of susceptible mice strains, which would otherwise succumb to infection following ST-OVA (46). Cross-presentation can also occur when phagosomal antigens are shunted into the cytoplasm for proteosomal antigen processing (22, 25). By utilizing the ST-OVA-C model, I may be facilitating the cross-presentation pathway. However, further molecular characterization is required to confirm this. By modulating the location of antigen during infection (phagosomal vs cytoplasmic) I was able to modulate the kinetics of antigen processing, presentation and subsequent CD8⁺ T cell response. Both strains of ST have similar virulence factors, express similar levels of OVA, and infect APCs similarly (46) (**Fig. 16, 17**). By utilizing both recombinants of ST

expressing OVA, I was able to determine the impact of antigen location and antigen presentation on DC function.

DCs are highly phagocytic sentinel immune cells that acquire invading pathogens in various tissue environments, allowing them to engage inflammation, antigen presentation and even cell death to promote immune protection. Thus DCs represent an important immunological link between pathogen detection and induction of protective adaptive immune response (51). DCs exist in two states; immature patrolling cells acquiring pathogenic antigen or mature APCs presenting processed antigen (406). DCs also have the ability to engage different pathways of cell death to facilitate an appropriate inflammatory or non-inflammatory environment for proper immune control of a pathogen. DCs also specialize in various mechanisms of antigen presentation by favouring antigen presentation over pathogen destruction (28). CD8 α DCs maintain a higher pH in the phagosome (111, 214). This promotes processing of phagosomal antigens through the cross-presentation pathway and loading onto MHC class-I molecules (111). Interestingly, when the number of CD8 α DCs are increased within the host by administration of FLT3L, the host can succumb to infection following intravenous injection of LM (407). This can be a consequence of CD8 α DCs favouring antigen presentation over pathogen elimination (407). By allowing pathogens to persist in the phagosome, CD8 α DCs can also be critical for mediating inflammatory cytokine and promote antigenic availability. Pathogen recognition and enhanced uptake through phagocytosis facilitates DCs to mature into professional APCs, allowing them to migrate into draining lymph nodes and

activate naïve T cells (406). In stark contrast, macrophages maintain a harsh environment in the phagosome, rapidly destroying pathogens and protecting the host from an escalation in pathogenic burden (55). However, macrophages are highly capable of pathogen elimination through innate mechanisms and are critical for immune control of bacterial burden (53, 55). Indeed my data indicates that BMMs infected with ST require a higher MOI than GM-CSF-DCs before cell death is induced, which is indicative of their higher capacity of pathogen control (**Fig. 44**). Thus, to address the impact of various immunological processes such as antigen presentation, inflammation and cell death on immune protection against ST, I focused on DCs because they are the most effective APC population that induces a robust CD8⁺ T cell response.

IFN-I is a pleiotropic cytokine expressed ubiquitously by most cells. Its primary role was thought to be an anti-viral cytokine that is critical for immunity against various viral pathogens (408). The role of IFN-I in cellular immunity has since expanded to include antigen-processing, migration, cell death, adaptive immunity and also immune exhaustion (190, 193). IFNAR has been shown to be critical for cross-presentation against soluble, viral and tumor antigens in DCs (190, 409). Thus the impact of IFNAR on antigen presentation and priming CD8⁺ T cells against ST was a question of high interest. My results indicate that IFN-I signalling is critical to mount a CD8⁺ T cell response against ST (**Fig. 14, 18**). However, this impact was independent of cross-presentation as both ST-OVA and ST-OVA-C yielded lower CD8⁺ T cell responses in IFNAR1-deficient mice (**Fig. 14, 18**). The impact of IFN-I signalling on cross-presentation was to

promote antigen retention and maintaining antigenic integrity for MHC class-I cross-presentation (111, 410). DCs can also promote antigen presentation and T cell immunity by providing antigen load to lymphoid organs to promote antigen transfer and processing by other resident DCs (107). Batf3-dependent CD8 α and CD103⁺ DCs have been shown to be the major cross-presenting APCs *in-vivo* (82, 101, 106, 411). Furthermore, both resident CD8 α and migratory CD103⁺ DCs have been shown to be paramount during infection with various pathogens by promoting early cytokine production, cross-presentation and priming of CD8⁺ T cells (101, 107, 109, 122, 409, 412, 413). My data indicates that migratory CD103⁺ DCs are able to engage ST mediated cell death following infection with high MOI, which is reduced in IFNAR1-deficient DCs (**Fig. 22**). IFNAR signalling has been shown to mediate necroptosis following ST infection in macrophages through engagement of TLR4 and ISGF3 signalling pathways (231, 414). In addition, IFN-I signalling can also lead to apoptotic cell death (415). Therefore, IFN-I signalling can impact many facets of immunity, and examining the impact of IFN-I on modulating immunity against ST was an area of high interest.

My results indicate that IFNAR mediated signalling is critical for DCs to promote CD8⁺ T cell responses against ST (**Fig. 14, 18**). This impact was not observed with systemic ST infection (**Fig. 15, 19**), which indicates that IFN-I signalling is critical when APC have to migrate and transport the antigen to the draining lymph nodes. The ability of IFNAR signalling to promote CD8⁺ T cell responses in the draining lymph nodes is most likely attributed to its influence on inflammation and cell death (**Fig. 22-24**), and less on its impact to modulate

antigen presentation (**Fig. 12, 15, 19**). Furthermore, my data suggests that IFNAR signalling is critical for CD8⁺ T cell responses against ST in the lymph nodes (**Fig. 18, 25**). Indeed, it would be tempting to speculate that migratory DCs capture ST antigen from peripheral tissue and migrate to the draining lymph nodes, effectively transporting antigen to the T cell zones and promoting CD8⁺ T cell responses. In this environment, antigen would be transferred from migratory CD103⁺ DCs to resident DCs, a phenomenon observed with other pathogens (416, 417). This can be achieved by promoting IFN-I mediated cell death in CD103⁺ DCs, which would ultimately be a source of cellular antigen capable of engaging cross-presentation and priming of CD8⁺ T cell responses (94, 113, 418). An alternative mechanism of antigen transfer may occur through cross-dressing, whereas intact MHC class-I-peptide complexes are transferred between cells without the requirement of further antigen-processing (419, 420). Following arrival in the lymph nodes, my data suggests that infected migratory DCs must engage IFN-I mediated inflammation, cytokine release (**Fig. 23-24**) and cell death (**Fig. 22**) to promote antigen presentation and engage a protective CD8⁺ T cell response against ST (**Fig. 18**).

Migratory subsets of DCs transit from the site of infection and lymphoid organs to mount a CD8⁺ T cell response against invading pathogens. My results suggest that cross-presenting CD103⁺ DCs are critical for immune protection against ST (**Fig. 34**). However, their particular role in the kinetics of mounting a CD8⁺ T cell response is still unclear. CD103⁺ DCs can acquire ST from the site of infection, activate and migrate to lymphoid organs (107). However, it is not clear

if this population of migratory DCs are the ones that mediate antigen presentation to CD8⁺ T cells. Alternatively, these infected migratory DCs can undergo appropriate cell death in the draining lymph nodes, and transfer antigen to other resident DCs, through a process called efferocytosis (94, 113, 221). In contrast, some pathogens require DCs to turn off cell death mechanisms to promote antigen presentation (107). For instance, influenza uptake by migratory CD103⁺ DCs is favoured during pulmonary infection, due to the enhanced uptake and intracellular replication within CD103⁺ DCs, which allows this by turning off interferon signalling, a critical antiviral cytokine (107). CD103⁺ DCs allow persistent intracellular replication of influenza while these migratory DCs traffic to the draining lymph node (107). CD103⁺ DCs can induce cell death due to high viral load, which allows availability of influenza antigens in the lymph nodes that can be transferred to resident DC subset in the lymph nodes that can mediate antigen presentation to naïve CD8⁺ T cells (107). Similar function has also been associated with a specialized subset of macrophages called CD169 macrophages against vesicular stomatitis virus (VSV) infection (211).

IFN-I mediated death of ST infected DCs may be critical to mount a CD8⁺ T cell response. My data suggests that migratory DCs undergo inflammatory cell death in a process that is dependent on IFNAR signalling (**Fig 22**). IFNAR1-deficient CD103⁺ DCs are unable to engage cell death (**Fig. 22**), which correlated with the lack of an effective CD8⁺ T cell response (**Fig. 18**). This may be the result of antigen transfer from migratory CD103⁺ DCs to resident CD8 α DCs in the lymph nodes. By allowing ST infected DCs to migrate to the lymph nodes

(**Fig 25**), and subsequently engage in IFN-I mediated cell death (**Fig. 22**), ST antigen becomes available in relevant compartments of lymphoid organs. In this environment, transfer of antigen from infected cells to CD8 α DCs, which are highly capable of mounting a CD8⁺ T cell response through cross-presentation, can facilitate antigen acquisition and antigen presentation to CD8⁺ T cells. Furthermore, this impact was more apparent with migratory CD103⁺ DC subsets and less with resident CD8 α DCs. This indicates that cell death of migratory DCs may influence antigen uptake by CD8 α DCs and promote antigen presentation. Furthermore, CD8 α DCs are maintained in areas of the lymph nodes that are in close proximity to naïve CD8⁺ T cell, thereby further facilitating a cytotoxic CD8⁺ T cell response (421). Altering the ability to undergo cell death or engaging an inappropriate cell death pathway can influence the kinetics of the CD8⁺ T cell response against ST and subsequent cell death. Thus, my data suggests that DCs regulate both inflammation and cell death to mediate immunity against ST.

DCs represent a heterogeneous group of immune cells, each with a distinct ontogeny and immune function during a variety of immune responses against pathogens (52). CD8 α DCs are specialized cross-presenting APCs that are highly relevant against pathogens and tumour cells *in-vivo* (101). Batf3 is an AP-1 transcription factor critical for the development of CD8 α DCs (101). Batf3-deficient mice are devoid of any CD8 α DCs at steady state, while maintaining proper development of all other DC subsets (101). Previously, mice deficient in other key transcription factors, such as IRF8 were used to evaluate the role of CD8 α DCs (195, 422). Interestingly, Batf3 has been shown to potentiate the

impact of IRF8 to maintain the development of the CD8 α DC lineage (423). Using the Batf3-deficient mouse model, CD8 α DCs have been shown to be critical for early production of IL-12 cytokine, which mediates immune production against *Toxoplasma gondii* (109). Other intracellular pathogens such as *Leishmania major* also require CD8 α DCs for protective immunity (412). Interestingly, LM, an intracellular Gram-positive bacterium, utilizes CD8 α DCs to traffic within the spleen (122). CD8 α DCs are critical for LM induced pathology, as LM is unable to induce systemic infection in Batf3-deficient mice (122). My data suggests that CD8 α DCs are critical for immune protection against ST. Both resistant and susceptible mouse strains deficient in Batf3 exhibit delayed CD8 $^{+}$ T cell responses (**Fig. 28-31**). This resulted in higher bacterial burden and enhanced mortality of Batf3-deficient mice (**Fig. 33**). This impact was also shown with a localized infection of ST, whereas Batf3-dependent CD103 $^{+}$ DCs play a similar role in the periphery (**Fig. 34**). Interestingly, ST did not utilize CD8 α DCs to traffic into different lymphoid organs as Batf3 $^{-/-}$ mice had enhanced bacterial burden (**Fig. 34**). This is in stark contrast to LM, which does not induce an effective infection in mice that lack CD8 α DCs (122). Indeed CD8 α DCs have multiple immune mediated roles against a variety of pathogens from cytokine production to antigen availability. Certain pathogens such as ST can eliminate CD8 α DCs during infection (**Fig. 27**) (424). However, backup mechanisms such as those mediated by Batf and Batf2 are in place to maintain CD8 α DC numbers, which are critical for immune protection against a variety of pathogens (**Fig. 32**) (381).

DCs can phagocytose particulate matter, and therefore can efficiently pick up ST even in the absence of virulence factors (425). The key question I wanted to address was why CD8 α DCs are so critical for immune protection against ST. Are they more capable of acquiring antigen from ST or are they more specialized at antigen presentation of pathogens such as ST. I noted that CD8 α DCs had higher ST infection at 2 hours post-infection in comparison to CD11b DCs. This can be due to the delayed acidification of phagosomes to limit protease activity within CD8 α DCs, a mechanism used by this cell type to process antigens for cross-presentation (215). Alternatively, it is conceivable that the CD8 α DCs are endowed with a better mechanism of phagocytosis of ST. Studies have shown that both CD8 α and CD11b DCs are able to pick up similar amounts of antigen, however processing of antigen varies greatly amongst the two subsets of conventional DCs (426). However, other studies have shown that CD8 α DCs display enhanced uptake of particulate antigen such as dead cells (118), but not inert beads (426). It is also possible that enhanced uptake of ST by CD8 α DCs can be the result of unique host receptor mediated mechanism. My results indicate that CD8 α DC is necessary for early CD8⁺ T cell priming of phagosomal bacteria irrespective of the antigenic location (phagosome or cytosol) (**Fig 28-31**). Furthermore, CD8 α DCs are an important source of inflammatory cytokines and chemokines, which facilitate DC maturation and chemo-attraction of various immune cells (**Fig. 16, 23, 24, 35, 36, 44-46**). MIP-1 α is a critical chemokine for recruitment of DC precursors into circulation and activation of immature DCs by binding to CCR1 and CCR5 expressed on DCs (427). IL-1, TNF α and IL-6 are

critical inflammatory cytokines that induce DC maturation and consequent expression of inflammatory chemokines such as MIP-1 α (428). By virtue of these soluble factors, other DC subsets can become readily activated (429) and help control bacterial burden *in-vivo*.

Furthermore, I wanted to address how antigen location can modulate the function of Batf3-dependent DCs to promote CD8⁺ T cell responses against ST. The impact of Batf3 was profound when utilizing the recombinant bacteria that translocate antigen into the cytoplasm (ST-OVA-C) (**Fig 30-31**) as opposed to when antigen remains in the phagosome (ST-OVA) (**Fig 28-29**). These results can be explained by the relative timing of antigen presentation that occurs upon infection with the two recombinants. In case of phagosomal antigen, the CD8⁺ T cell response is delayed (**Fig. 9-11**), and hosts die even before the impact of Batf3 can be realized (**Fig. 28C-D**) (161). On the other hand, cytosolic antigenic delivery results in better survival of wild-type mice due to induction of potent CD8⁺ T cell response (46), and any delay in this activation compromises host survival (**Figure 33**). This recombinant strain of ST that deposits antigen in the cytoplasm of infected cells (ST-OVA-C) is readily degraded by the proteasome and processed for presentation to CD8⁺ T cells (direct-presentation) (46). This is an interesting result as it suggests a critical role for CD8 α DCs against cytoplasmic antigen with inflammatory signals originating from the phagosome, which are relevant for pathogens that shed toxins (antigens) into the cytoplasm from the phagosome such as *Salmonella* or *Shigella* (430). This can also suggest that ST-OVA-C is cross-presented in CD8 α DCs by the cytosolic pathway that

requires proteosomal processing (28). My study also highlights that the impact of Batf3 is not universal in all intracellular bacterial infection models as LM induced poor infection in Batf3-deficient mice (122), whereas in the case of ST the infection is exacerbated (**Fig. 29, 32, 33**). Furthermore, CD8 α DCs are critical for trafficking of LM in the spleen and inducing a productive infection (122). My results indicate that ST does not need to actively infect CD8 α DCs to initiate infection as the ST burden in the spleens was similar in wild type and Batf3-deficient mice during the initial time points that are critical for antigen presentation (**Fig. 32C**). Batf3 has been shown to promote CD8 $^+$ T cell response in a viral and solid tumour model (101). I have shown that Batf3 plays an important role in promoting CD8 $^+$ T cell response against a phagosomal pathogen, but the significance of the impact is realized only when antigen processing is rapidly induced through the cytosolic pathway. Collectively, these results provide significant insights into vaccine development approaches wherein engagement and activation of CD8 α DC is important irrespective of the nature of the vaccine such as live, subunit, cytosolic or phagosomal.

The induction of cell death has been shown to promote antigen presentation, in particularly cross-presentation mediated by DCs through a process called efferocytosis (94, 113, 221, 431). Classically, apoptosis has been viewed as a form of cell death that induces limited inflammation and provides antigenic cargo in the form of apoptotic bodies or blebs (432). Following the release of apoptotic bodies, the apoptotic cell releases soluble factors such as fractalkines, ATPs and uridine triphosphates (UTPs) to promote clearance of the

antigenic cargo, essentially a “find-me” signal to recruit phagocytes (432-434). Furthermore, apoptotic bodies expose phosphatidylserine on the surface to promote phagocytosis, and thus indirectly providing antigen to phagocytic cells that also act as APCs (432, 435, 436). This is in stark contrast to necrosis, which is a form of accidental cell death engaged following trauma or infection, which results in the release of cellular contents and the induction of inflammation (437). Cells that have undergone apoptosis following Bacillus Calmette-Guérin (BCG) infection have been shown to provoke a robust CD8⁺ T cell response by providing antigen through availability of apoptotic bodies to nearby circulating DCs (382). Interestingly, RipK3 has also been shown to promote antigen presentation following cell death that relies on an NF-κB dependent pathway (438). My results indicate that during ST infection of macrophages and DCs, cell death is engaged preferably through RipK3 mediated necroptosis and Caspase1/11 mediated pyroptosis (**Fig. 44**). Furthermore, my data suggest apoptosis may not be a critical pathway engaged during ST infection as inhibition of pan-Caspases by treating cells with zVAD-fmk actually potentiates RipK3 mediated necroptosis and release of inflammatory cytokines (**Fig. 50**). Furthermore, even at higher MOIs of infection, RipK3-Caspase1/11-deficient macrophages and DCs cannot engage cell death following ST infection (**Fig. 44**). This suggests both Caspase1/11 and RipK3 mediate critical cell death pathways that are engaged following ST infection.

When approaching the central question of how Caspase1/11 and RipK3 impact CD8⁺ T cell responses against ST, I considered the strong possibility that

cell death can provide both antigen availability and inflammation, which would induce a robust CD8⁺ T cell response. Thus mice lacking either Caspase1/11 or RipK3 cell death pathways would induce a muted CD8⁺ T cell in response to ST infection. To my surprise, there was no impact on priming of CD8⁺ T cell responses against ST when disabling Casase1/11 (**Fig. 39C**) and RipK3 (**Fig. 40F**) cell death pathways. Albeit, there was even slightly higher CD8⁺ T cell responses in Caspase1/11-deficient mice following ST infection (**Fig. 40F**), which is most likely a result of increased bacterial burden (and increased antigen availability) at day 7 when the CD8⁺ T cell response was measured (**Fig. 39D, 42A, 42D**). Thus I was able to take a novel approach at deciphering the critical role of Caspase1/11 and RipK3 during ST infection by utilizing ST-OVA-C. The recombinant strain ST-OVA-C induces a potent CD8⁺ T cell response within the first week of infection (46). By engaging early CD8⁺ T cell response against ST, I was able to reveal the critical role of Caspase1/11 and RipK3 in promoting bacterial clearance through inflammation and cell death. Engaging a robust CD8⁺ T cell response within the first week of infection also allowed the mice to survive long enough to decipher the impact of cell death pathways on bacterial burden and host mortality following ST infection. This was somewhat paradoxical as in most cases; the innate immune response promotes adaptive immune responses. In my study, the adaptive immune response (mediated by the CD8⁺ T cell response) allowed for the ideal conditions to decipher the critical role of Caspase1/11 and RipK3 in promoting innate immune defences against ST.

Engagement of proper cell death pathways following infection is critical to the host defense against various pathogens. For instance, RipK3 mediated necroptosis and Caspase-1 mediated pyroptosis have been shown to be employed as a defense mechanism to impede viral dissemination within a given host (216, 255, 320). Despite these findings, the role of inflammatory cell death such as necroptosis and pyroptosis during virulent ST infection is still unclear, partly due to the rapid fatality of RipK3- and Caspase1/11-deficient mice, which are bred onto the B6 background (175, 439, 440). The molecular mechanisms that govern the induction and execution of pyroptosis and necroptosis are quite distinct (320, 441). However, activation of both cell death pathways results in the release of various DAMPs following membrane rupture, which leads to excessive inflammation (216, 442). Induction of inflammation has become synonymous with engagement of pyroptotic cell death (443), as Caspase-1 cleavage of inflammatory cytokines precedes cell lysis (320). However, the association of RipK3 mediated necroptosis in promoting inflammation is a new area of interest (216, 269, 444).

Caspase1/11-signalling is an important mechanism of cell-death to mediate immune protection following ST infection (163, 175, 333, 440). Furthermore, it was shown that RipK3-deficient macrophages also undergo reduced cell-death following infection with ST (231). Previously, the increase in bacterial burden in NLRC4-NLRP3 double-deficient mice was shown to be similar to Caspase1/11-deficient mice, suggesting that NLRC4 and NLRP3 inflammasomes play a redundant role in promoting the control of ST (176).

However, my results indicate that Caspase1/11 and RipK3 play synergistic roles in facilitating inflammasome activation, as Caspase1/11-RipK3 double deficient mice exhibit increased susceptibility to systemic ST infection in comparison to wild type or single Caspase1/11 and RipK3 knockout mice (**Fig. 42A-E**). This data was surprising, as it suggests that both Caspase1/11 and RipK3 signalling molecules synergize to mediate comprehensive control of the bacterium. Thus, RipK3 must mediate protection by mechanisms that are in addition to NLR activation in order for Caspase1/11-Rip3-double deficient mice to show enhanced susceptibility in comparison to Caspase1/11-deficient mice. Furthermore, my data provides *in-vivo* evidence for the critical role of Caspase1/11 (**Fig. 39**) and RipK3 (**Fig. 40D-F**) during systemic ST infection by utilizing the recombinant ST-OVA-C. In addition, I also backcrossed both Caspase1/11 and RipK3 on the B6.Nramp^{G169} mouse strain to best address the role of the aforementioned cell death adaptors during ST-OVA infection. I observed a similar requirement of Caspase1/11 and RipK3 for protection against ST-OVA (**Fig. 41A-C**). Thus my data suggests that both Caspase1/11 and RipK3 cell death adaptors are critical for immune protection against ST irrespective of antigen location during ST infection.

The inflammasome can be engaged by both the canonical and non-canonical mechanisms of activation (238, 343, 445, 446). Canonical activation of inflammasome is mediated when Caspase-1 employs the recruitment of NLRs, ASC to form the multiprotein complex, which processes IL-1 cytokines into their active form (238). Pyroptosis is also engaged by Caspase-1 mediated canonical

inflammasome activation, which facilitates release of processed IL-1 cytokines, which act as DAMPs following release from the cell (320). Kayagaki et al. had recently shown that the original Caspase-1 deficient mice also have a deficiency in Caspase-11 as a result of generating the original knockout mouse by utilizing 129 embryonic stem cells, which maintains a 5 base pair deletion within the *Caspase-11* gene, resulting in a premature stop codon and a non-functional Caspase-11 protein (445). Since both Caspase-1 and Caspase-11 are encoded in close proximity in chromosome 9, both mutations were co-segregated during backcrossing onto the B6 background. Interestingly, Kayagatki et al. generated a Caspase-1 deficient mouse by restoring expression of Caspase-11 using a bacterial artificial chromosome to confirm Caspase-1 is critical for IL-1 β production following canonical inflammasome activation (445). Interestingly, Caspase-11 deficiency resulted in protection against LPS shock, when administered at lethal doses, indicating non-canonical Caspase-11 mediated inflammasome is critical for inflammatory responses against LPS (445). Additionally, Broz et al. utilized Caspase-11-deficient and Caspase-1^{-/-}Caspase-11^{Tg} and determined the role of various inflammatory caspases during ST infection (447). The authors show that Caspase-1-deficient mice are more susceptible to ST infection than Caspase1/11-deficient mice (447). However, since the authors utilized B6 inbred mice (which rapidly succumb to infection following ST infection) the differences in bacterial burden was quite modest (447). Furthermore, recent studies have shown that Caspase-11 mediated non canonical activation of inflammasome rely on IFN-I signalling for guanylate

binding protein (GTPases) to detect Gram negative bacteria (448, 449). Interestingly, Caspase-11 can engage lysis of the cell that is independent of processed IL-1 cytokine production, which is reserved for Caspase-1 mediated inflammasome activation (450). Paradoxically, while Caspase-1 mediated pyroptosis is protective against ST, engaging Caspase-11 mediated cell death is detrimental for host survival (445, 450). This indicates that Caspase-11 mediated lysis acts in concert with pyroptosis to promote immune defense while Caspase-11 mediated lysis, when engaged alone is detrimental to host defenses (445). In my model, I utilized the Caspase1/11-deficient mouse model which disables both canonical and non-canonical pathways of inflammasome activation to evaluate the role of these inflammatory cytokines during ST infection (353, 445). I have shown that canonical and non-canonical pathways of inflammation are critical for immune protection against ST (**Fig. 39, 41**). Furthermore, Caspase1/11 was critical for control of bacterial burden and production of IL-18 (**Fig. 39, 41**), which was protective when administered during ST infection (**Fig. 57**). Indeed, my approach utilized the original Caspase-1 mouse model (353), which disables both Caspase-1 and Caspase-11 mediated inflammasome activation to decipher the mechanism of immune protection against ST (445).

Inflammation often precedes induction of cell death. Furthermore, there is growing evidence suggesting a complex link between the induction of cell death and processing of pro-inflammatory cytokines (216, 442). Since Caspase1/11 and RipK3-deficient mice were more susceptible to ST infection (**Fig. 39-42**), the mechanism of this susceptibility may be due to an impairment of inflammation as

opposed to their defects in cell death. To investigate this further, I utilized *in-vitro* cultures of BMMs and GMCSF-DCs and examined the role of Caspase-1, Caspase-8 and RipK3 in the processing of pro-inflammatory cytokines during ST infection. My results indicate that cell-death by Caspase1/11 is more dominant in comparison to that induced by RipK3 (**Fig. 44A, C**). Furthermore, Caspase1/11-RipK3 double-deficient macrophages are even more resistant to cell death (**Fig. 44A, C**). Lower IL-1 cytokine production in Caspase1/11- and RipK3-deficient mice following ST infection further corroborated these results. I showed that BMMs and GMCSF-DCs deficient in RipK3 and Caspase1/11 secreted reduced levels of IL-1 α , IL-1 β and IL-18 following ST infection (**Fig. 44B, D**). By disabling distinct cell death pathways in RipK3^{-/-} and Caspase1/11^{-/-} cells, secretion of IL-1 cytokines (such as IL-1 β) will be hampered as these “alarmins” are released from dying cells (396). Immune cells deficient in RipK3 and Caspase1/11 are unable to induce cell death upon ST infection (**Fig. 44A, C**). This suggests alternative cell death pathways such as apoptosis are not generally engaged following ST infection. Furthermore, by utilizing pan-Caspase inhibitor zVAD, I was able to induce significant levels of RipK3 mediated cell death (217) in ST infected BMMs and GMCSF-DCs, suggesting necroptosis as a major mechanism of cell death following ST infection (**Fig. 50A, D**). As expected, this impact was not evident in RipK3^{-/-} cells (**Fig. 50A, D**).

Caspase-1 mediated processing and release of IL-1 β and IL-18 has been shown to promote protection against various pathogens (175). Furthermore, Caspase-1 mediated cell death also promotes immune protection by releasing

intracellular bacteria within infected cells for uptake and degradation by neutrophils, independent of IL-1 β and IL-18 production (333). Following cell rupture, any non-processed pro-IL-1 β and pro-IL-18 could also be potentially processed by extracellular proteases (282). Furthermore, RipK3 also promotes IL-1 β processing through various mechanisms involving Caspase-8 and cIAPs (237, 389, 451). Thus it was of great interest to evaluate the impact of inflammatory IL-1 cytokines IL-1 β and IL-18 on inflammation and immune responses that promote protection against ST. To address this issue, I administered recombinant murine IL-1 α/β and IL-18 *in-vivo* to ST infected Caspase1/11^{-/-} and RipK3^{-/-}Caspase1/11^{-/-} mice (**Fig. 57**) that generally exhibit high bacterial burden within the first week of infection (**Fig. 42C, D**). My results indicate that secretion of processed IL-18 is the critical protective mechanism following Caspase1/11-Rip3 signalling (**Fig. 57**). In addition, cells deficient in Caspase1/11 and RipK3 were not able to produce any IL-18 in both *in-vitro* and *in-vivo* models following ST infection (**Fig. 44, 47**). Caspase1/11-Rip3-deficient mice are unable to process pro-IL-18 into its bioactive form (**Fig. 47**), which may explain the exacerbation in ST infection *in-vivo* at late stages of infection (**Fig. 42C, D**). In addition, bacterial burden in Caspase1/11-Rip3-deficient mice was significantly reduced by administering recombinant IL-18 *in-vivo* (**Fig. 57**). My results also revealed an interesting observation where Caspase1/11-deficient mice treated with recombinant IL-1 β led to exacerbation, rather than control of infection (**Fig. 57**). The reason for this paradoxical impact of IL-1 β in Caspase1/11-deficient mice is not clear. It is possible that IL-1 β acts as a potent

DAMP, which may have a deleterious impact selectively in Caspase1/11-deficient mice, where the bacterial burden and consequent inflammation is already high (**Fig. 42**). By shutting down two critical death pathways engaged following ST infection, I drive the system to necrosis at late stages of infection, resulting in uncontrolled release of DAMPs (IL-1 α/β) that results in massive inflammation.

My results have revealed that there is significant redundancy in inflammasome activation by different bacterial PAMPs, and that there are significant differences between macrophages and DCs in terms of their dependence on flagellin for inflammasome activation (**Fig. 53**). While Caspase-1 was identified initially as the principal driver of inflammasome activation, recent reports have suggested that Caspase-8 signalling also mediates the activation of inflammasomes (285, 387, 452, 453). Recently, members of the Ripoptosome complex have been shown to promote inflammasome activation (389, 452). In contrast to this, Caspase-8 was shown to limit NLRP3 inflammasome activation, which was dependent on various members of the necrosome (451). Indeed, regulation of RipK3-dependent necrosis by Caspase-8 has been considered as an essential mechanism for maintenance of homeostasis, as RipK3-deficiency rescues the embryonic lethality of Caspase-8-deficient mice (257, 258). My data suggests that Caspase-1 negatively regulates Caspase-8 (**Fig. 52**) to promote its dominance in inflammasome activation and IL-1 cytokine production. Furthermore, my results have uncovered significant redundancy in inflammasome signalling, as Caspase-8 signalling becomes critical only in response to flagellin in the absence of Caspase1/11 and RipK3 (**Fig. 53, 58, 59**).

Processing of IL-1 β was initially thought to rely on Caspase-1 mediated activation of the inflammasome (238). However recent publications had indicated a critical role of RipK3 (237) and Caspase-8 (451) in cytokine processing of IL-1 β . Thus I examined the regulation of IL-1 β during ST infection by utilizing various inhibitors of caspases and RipKs. My results revealed that there are several checkpoints that regulate inflammasome activation (**Fig. 58**). RipK3 promoted Caspase-8-dependent inflammasome activation, however, this interaction was regulated by the kinase region of RipK3 (**Fig. 54**). Surprisingly, inhibition of the RipK3 kinase region unleashed massive Caspase-8 dependent inflammasome activation and produce excessive amounts of IL-1 β (**Fig. 54**). There are conflicting reports regarding activation (388) or inhibition (389) of Caspase-1 and IL-1 β production by cIAPs. My results indicate that cIAPs regulate inflammasome activation at various steps (**Fig. 59**), and interestingly, Caspase-1 appears to regulate the expression of cIAPs and Caspase-8-processing, thereby explaining why Caspase-1 plays a dominant role in inflammasome activation (**Fig. 58**). Regulation of ST mediated IL-1 β production is critical for an optimal immune response against ST, which can be exploited for therapeutic use by regulating cell death or IL-1 β processing checkpoints.

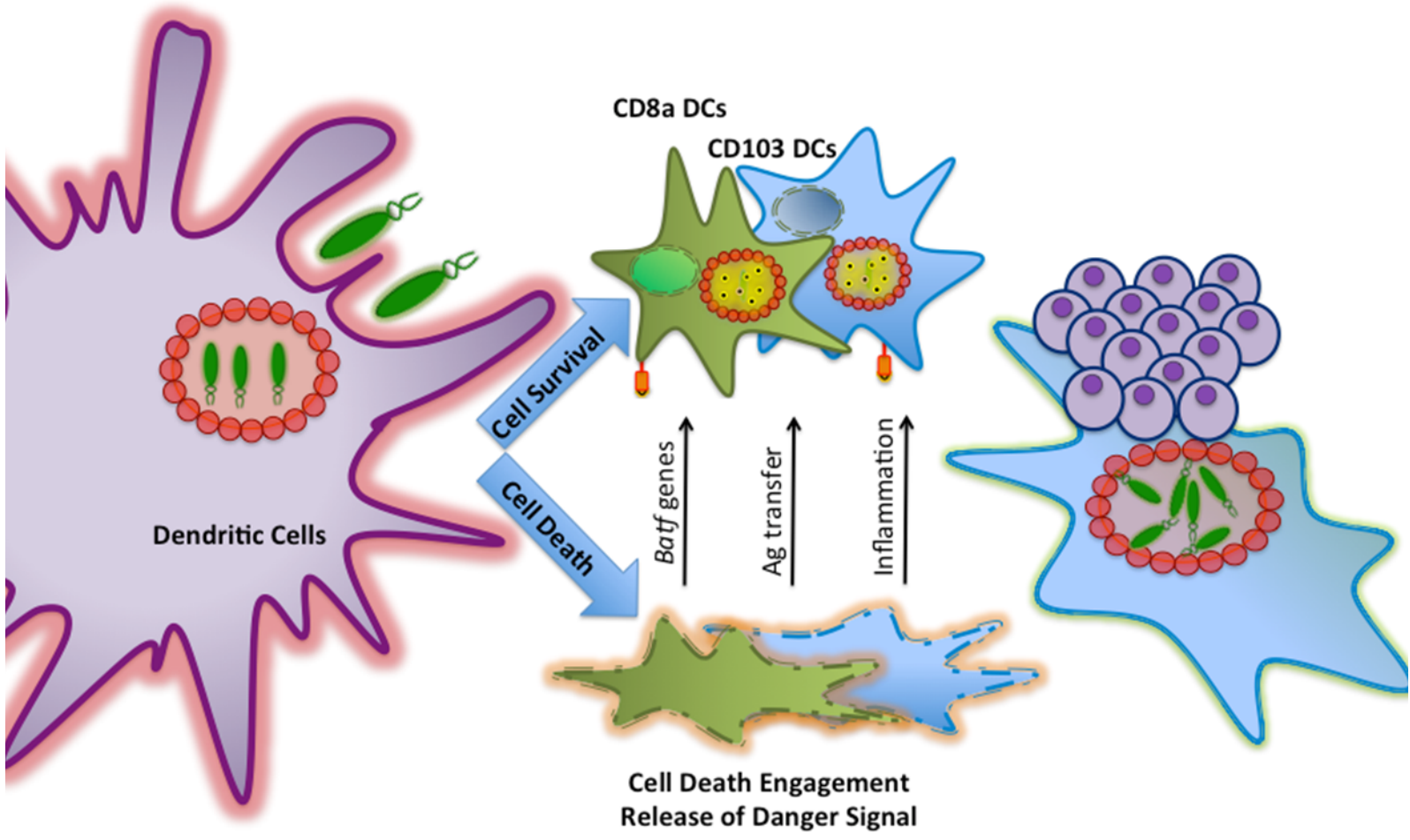
Overall, my data shows that ST can promote inflammatory cell death through pyroptosis and necroptosis, which can lead to the release of danger signals and inflammatory cytokines. Furthermore, cellular antigen becomes more readily available following the death of ST infected cells, which can promote antigen processing and presentation. Additionally, inflammatory cytokines and

chemokines are secreted following infection with ST, which can result in an influx of additional immune cells to facilitate pathogen control and promote DCs to prime the adaptive immune response against ST. Indeed my data suggest that CD8⁺ T cells are critical for host immune protection against ST, which can be mediated by CD8 α DCs. Modulation of antigen presentation, inflammation and cell death can promote better pathogen control and host survival following ST infection, which can have implications for better vaccine design against chronic intracellular bacteria (**Fig. 60**).

There are no efficient therapeutics against phagosomal pathogens (Salmonella, Mycobacteria, Leishmania). Overall, my study unravels novel pathways that are exploited by ST such as antigen presentation, inflammation and cell death to induce immune evasion. The insights gained from the study outlined above may bear implication on the development of novel therapeutics and effective vaccine design against intracellular bacteria.

Figure 60: Thesis Overview

DCs are sentinel immune cells which mediate immune protection against ST. CD8 α and CD103⁺ DCs are specialized subsets that have enhanced capabilities of acquiring ST antigen and promoting cross-presentation and priming of CD8⁺ T cells. My data shows that ST can promote inflammatory cell death through pyroptosis and necroptosis, which can lead to the release of danger signals and inflammatory cytokines. Under inflammatory conditions, Batf and Bat2 transcription factors can be employed to work in concert with Batf3 and promote development of additional CD8 α DCs. Furthermore, cellular antigen becomes more readily available following the death of ST infected cells, which can promote antigen processing and presentation. Additionally, inflammatory cytokines and chemokines are secreted following infection with ST, which can result in an influx of additional immune cells to facilitate pathogen control and promote DCs to prime the adaptive immune response against ST. Indeed my data suggest that CD8⁺ T cells are critical for host immune protection against ST, which can be mediated by CD8 α DCs. Modulation of antigen-presentation, inflammation and cell death can promote better pathogen control and host survival following ST infection, which can have implications for better vaccine design against chronic intracellular bacteria.



5: COPYRIGHT PERMISSION

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 29, 2015

This is a License Agreement between Rajen Patel ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3718211235206
License date	Sep 29, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo
Licensed content author	José A. Villadangos, Petra Schnorrer
Licensed content date	Jul 1, 2007
Volume number	7
Issue number	7
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Fig 1 - Antigen Presentation
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	DENDRITIC CELLS ARE KEY MEDIATORS OF PROTECTIVE IMMUNITY AGAINST SALMONELLA TYPHIMURIUM BY REGULATING ANTIGEN PRESENTATION, INFLAMMATION AND CELL DEATH
Expected completion date	Sep 2015
Estimated size (number of pages)	300
Total	0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

Note: For republication from the *British Journal of Cancer*, the following credit lines apply.

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.

Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK:

[JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

Note: For translation from the *British Journal of Cancer*, the following credit line applies.

Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 29, 2015

This is a License Agreement between Rajen Patel ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3718211439325
License date	Sep 29, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny
Licensed content author	Martin Guilliams, Florent Ginhoux, Claudia Jakubzick, Shalin H. Naik, Nobuyuki Onai, Barbara U. Schraml
Licensed content date	Jul 18, 2014
Volume number	14
Issue number	8
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Fig 2 - MPS Subsets
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	DENDRITIC CELLS ARE KEY MEDIATORS OF PROTECTIVE IMMUNITY AGAINST SALMONELLA TYPHIMURIUM BY REGULATING ANTIGEN PRESENTATION, INFLAMMATION AND CELL DEATH
Expected completion date	Sep 2015
Estimated size (number of pages)	300
Total	0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

Note: For republication from the *British Journal of Cancer*, the following credit lines apply.

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.

Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK:
[JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

Note: For translation from the *British Journal of Cancer*, the following credit line applies.

Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK:
[JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 29, 2015

This is a License Agreement between Rajen Patel ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3718210898437
License date	Sep 29, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks
Licensed content author	Theresa L. Murphy, Roxane Tussiwand, Kenneth M. Murphy
Licensed content date	Jun 21, 2013
Volume number	13
Issue number	7
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Transcriptional factors for DC development.
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	DENDRITIC CELLS ARE KEY MEDIATORS OF PROTECTIVE IMMUNITY AGAINST SALMONELLA TYPHIMURIUM BY REGULATING ANTIGEN PRESENTATION, INFLAMMATION AND CELL DEATH
Expected completion date	Sep 2015
Estimated size (number of pages)	300
Total	0.00 CAD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

Note: For republication from the *British Journal of Cancer*, the following credit lines apply.

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication) For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.

Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK:

[JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

Note: For translation from the *British Journal of Cancer*, the following credit line applies.

Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 29, 2015

This is a License Agreement between Rajen Patel ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3718220082508
License date	Sep 29, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Microbiology
Licensed content title	Persistent bacterial infections: the interface of the pathogen and the host immune system
Licensed content author	Denise M. Monack, Anne Mueller, Stanley Falkow
Licensed content date	Sep 1, 2004
Volume number	2
Issue number	9
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Fig. 5 - Salmonella Typhimurium
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	DENDRITIC CELLS ARE KEY MEDIATORS OF PROTECTIVE IMMUNITY AGAINST SALMONELLA TYPHIMURIUM BY REGULATING ANTIGEN PRESENTATION, INFLAMMATION AND CELL DEATH
Expected completion date	Sep 2015
Estimated size (number of pages)	300
Total	0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

Note: For republication from the *British Journal of Cancer*, the following credit lines apply.

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication) For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.

Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK:

[JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

Note: For translation from the *British Journal of Cancer*, the following credit line applies.

Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.



Note: Copyright.com supplies permissions but not the copyrighted content itself.

1
PAYMENT

2
REVIEW

3
CONFIRMATION

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us at +1.855.239.3415 Toll Free, M-F between 3:00 AM and 6:00 PM (Eastern), or write to us at info@copyright.com. This is not an invoice.

Confirmation Number: 11455410
Order Date: 09/29/2015

If you paid by credit card, your order will be finalized and your card will be charged within 24 hours. If you choose to be invoiced, you can change or cancel your order until the invoice is generated.

Payment Information

Order Details

Blood : journal of the American Society of Hematology

Order detail ID: 68480716
Order License Id: 3718271017262
ISSN: 1528-0020
Publication Type: e-Journal
Volume:
Issue:
Start page:
Publisher: AMERICAN SOCIETY OF HEMATOLOGY
Author/Editor: American Society of Hematology ; HighWire Press

Permission Status: **Granted**
Permission type: Republish or display content
Type of use: Thesis/Dissertation

Requestor type Academic institution

Format Electronic

Portion cartoon

Number of cartoons 1

Title or numeric reference of the portion(s) DCs can be cultured in-vitro...CD103i DCs (CD103+ DC equivalents).

Title of the article or chapter the portion is from Figure 8: In-vitro cultures of DCs

Editor of portion(s)	N/A
Author of portion(s)	N/A
Volume of serial or monograph	N/A
Page range of portion	1-300
Publication date of portion	September-December 2015
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	United States and Canada
In the following language(s)	Original language of publication
With incidental promotional use	no
Lifetime unit quantity of new product	Up to 499
Made available in the following markets	education
The requesting person/organization	Rajen Patel
Order reference number	
Author/Editor	Rajen Pate
The standard identifier	Thesis
The proposed price	0

Title	DENDRITIC CELLS MEDIATE PROTECTIVE IMMUNITY AGAINST SALMONELLA TYPHIMURIUM BY REGULATING ANTIGEN PRESENTATION, INFLAMMATION AND CELL DEATH
Publisher	University of Ottawa
Expected publication date	Oct 2015
Estimated size (pages)	300

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. [More info](#)

\$ 28.50

Total order items: 1	This is not an invoice.	Order Total:38.21 CAD (28.50 USD)
-----------------------------	--------------------------------	--

Confirmation Number: 11455410

Special Rightsholder Terms & Conditions

The following terms & conditions apply to the specific publication under which they are listed

Blood : journal of the American Society of Hematology

Permission type: Republish or display content

Type of use: Thesis/Dissertation

TERMS AND CONDITIONS

The following terms are individual to this publisher:

None

Other Terms and Conditions:

STANDARD TERMS AND CONDITIONS

1. Description of Service; Defined Terms. This Republication License enables the User to obtain licenses for republication of one or more copyrighted works as described in detail on the relevant Order Confirmation (the "Work(s)"). Copyright Clearance Center, Inc. ("CCC") grants licenses through the Service on behalf of the rightsholder identified on the Order Confirmation (the "Rightsholder"). "Republication", as used herein, generally means the inclusion of a Work, in whole or in part, in a new work or works, also as described on the Order Confirmation. "User", as used herein, means the person or entity making such republication.

2. The terms set forth in the relevant Order Confirmation, and any terms set by the Rightsholder with respect to a particular Work, govern the terms of use of Works in connection with the Service. By using the Service, the person transacting for a republication license on behalf of the User represents and warrants that he/she/it (a) has been duly authorized by the User to accept, and hereby does accept, all such terms and conditions on behalf of User, and (b) shall inform User of all such terms and conditions. In the event such person is a "freelancer" or other third party independent of User and CCC, such party shall be deemed jointly a "User" for purposes of these terms and conditions. In any event, User shall be deemed to have accepted and agreed to all such terms and conditions if User republishes the Work in any fashion.

3. Scope of License; Limitations and Obligations.

3.1 All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The license created by the exchange of an Order Confirmation (and/or any invoice) and payment by User of the full amount set forth on that document includes only those rights expressly set forth in the Order Confirmation and in these terms and conditions, and conveys no other rights in the Work(s) to User. All rights not expressly granted are hereby reserved.

3.2 General Payment Terms: You may pay by credit card or through an account with us payable at the end of the month. If you and we agree that you may establish a standing account with CCC, then the following terms apply: Remit Payment to: Copyright Clearance Center, Dept 001, P.O. Box 843006, Boston, MA 02284-3006. Payments Due: Invoices are payable upon their delivery to you (or upon our notice to you that they are available to you for downloading). After 30 days, outstanding amounts will be subject to a service charge of 1-1/2% per month or, if less, the maximum rate allowed by applicable law. Unless otherwise specifically set forth in the Order Confirmation or in a separate written agreement signed by CCC, invoices are due and payable on "net 30" terms. While User may exercise the rights licensed immediately upon issuance of the Order Confirmation, the license is automatically revoked and is null and void, as if it had never been issued, if complete payment for the license is not received on a timely basis either from User directly or through a payment agent, such as a credit card company.

3.3 Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) is "one-time" (including the editions and product family specified in the license), (ii) is non-exclusive and non-transferable and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Order Confirmation or invoice and/or in these terms and conditions. Upon completion of the licensed use, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period).

3.4 In the event that the material for which a republication license is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) which are identified in such material as having been used

by permission, User is responsible for identifying, and seeking separate licenses (under this Service or otherwise) for, any of such third party materials; without a separate license, such third party materials may not be used.

3.5 Use of proper copyright notice for a Work is required as a condition of any license granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Republished with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc. " Such notice must be provided in a reasonably legible font size and must be placed either immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

3.6 User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

4. Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

5. Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors and assigns.

6. Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

7. Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the license set forth in the Order Confirmation and/or these terms and conditions, shall be a material breach of the license created by the Order Confirmation and these terms and conditions. Any breach not cured within 30 days of written notice thereof shall result in immediate termination of such license without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

8. Miscellaneous.

8.1 User acknowledges that CCC may, from time to time, make changes or additions to the Service or to these terms and conditions, and CCC reserves the right to send notice to the User by electronic mail or otherwise for the purposes of notifying User of such changes or additions; provided that any such changes or additions shall not apply to permissions already secured and paid for.

8.2 Use of User-related information collected through the Service is governed by CCC's privacy policy, available online here: <http://www.copyright.com/content/cc3/en/tools/footer/privacypolicy.html>.

8.3 The licensing transaction described in the Order Confirmation is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the license created by the Order Confirmation and these terms and conditions or any rights granted hereunder; provided, however, that User may assign such license in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in the new material which includes the Work(s) licensed under this Service.

8.4 No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The

Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the licensing transaction described in the Order Confirmation, which terms are in any way inconsistent with any terms set forth in the Order Confirmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court. If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

v 1.1

Close

Confirmation Number: 11455410

Citation Information

Order Detail ID: 68480716

**Blood : journal of the American Society of Hematology by American Society of Hematology ; HighWire Press
Reproduced with permission of AMERICAN SOCIETY OF HEMATOLOGY in the format Thesis/Dissertation via
Copyright Clearance Center.**

Close

6: REFERENCE LIST

1. Hornef, M. W., M. J. Wick, M. Rhen, and S. Normark. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* 3: 1033–1040.
2. Medzhitov, R., and C. A. Janeway. 1997. Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* 9: 4–9.
3. Vivier, E., and B. Malissen. 2005. Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nat. Immunol.* 6: 17–21.
4. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat. Rev. Immunol.* 7: 543–555.
5. Janeway, C. A., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20: 197–216.
6. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394–397.
7. Barton, G. M., and J. C. Kagan. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat. Rev. Immunol.* 9: 535–542.
8. Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. *Cell* 140: 805–820.
9. Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* 30: 16–34.
10. Lamkanfi, M., and V. M. Dixit. 2012. Inflammasomes and Their Roles in Health and Disease. *Annu. Rev. Cell Dev. Biol.* 28: 137–161.
11. O'Neill, L. A. J., and A. G. Bowie. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* 7: 353–364.
12. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozören, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant, and G. Nuñez. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat. Immunol.* 7: 576–582.
13. Miao, E. A., D. P. Mao, N. Yudkovsky, R. Bonneau, C. G. Lorang, S. E. Warren, I. A. Leaf, and A. Aderem. 2010. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* 107: 3076–3080.
14. Matzinger, P. 1998. An innate sense of danger. *Semin. Immunol.* 10: 399–

415.

15. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Théry, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* 20: 621–667.
16. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *Journal of Experimental Medicine* 137: 1142–1162.
17. Tian, T., J. Woodworth, M. Sköld, and S. M. Behar. 2005. In vivo depletion of CD11c⁺ cells delays the CD4⁺ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. *J. Immunol.* 175: 3268–3272.
18. Jung, S., D. Unutmaz, P. Wong, G.-I. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman, and R. A. Lang. 2002. In vivo depletion of CD11c⁺ dendritic cells abrogates priming of CD8⁺ T cells by exogenous cell-associated antigens. *Immunity* 17: 211–220.
19. Blum, J. S., P. A. Wearsch, and P. Cresswell. 2013. Pathways of antigen processing. *Annu. Rev. Immunol.* 31: 443–473.
20. Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M. F. Princiotta, P. Thibault, D. Sacks, and M. Desjardins. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425: 402–406.
21. Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425: 397–402.
22. Rock, K. L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev.* 207: 1–18.
23. Kurts, C., B. W. S. Robinson, and P. A. Knolle. 2010. Cross-priming in health and disease. *Nat Rev Immunol* 10: 403–414.
24. Gutiérrez-Martínez, E., R. Planès, G. Anselmi, M. Reynolds, S. Menezes, A. C. Adiko, L. Saveanu, and P. Guermonprez. 2015. Cross-Presentation of Cell-Associated Antigens by MHC Class I in Dendritic Cell Subsets. *Front Immunol* 6: 363.
25. Kovacsovics-Bankowski, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267: 243–246.
26. Shen, L., L. J. Sigal, M. Boes, and K. L. Rock. 2004. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21: 155–165.
27. Lin, M. L., Y. Zhan, A. I. Proietto, S. Prato, L. Wu, W. R. Heath, J. A. Villadangos, and A. M. Lew. 2008. Selective suicide of cross-presenting CD8⁺ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. *Proc Natl Acad Sci USA* 105: 3029–3034.
28. Joffre, O. P., E. Segura, A. Savina, and S. Amigorena. 2012. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* 12: 1–13.
29. Kaech, S. M., E. J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* 2: 251–262.
30. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W.

- R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478–480.
31. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474–478.
32. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745–763.
33. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9: 765–775.
34. Kapsenberg, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3: 984–993.
35. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* 2: 415–422.
36. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naïve and effector T cells. *Immunity* 8: 89–95.
37. Jelley-Gibbs, D. M., N. M. Lepak, M. Yen, and S. L. Swain. 2000. Two distinct stages in the transition from naïve CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. *J. Immunol.* 165: 5017–5026.
38. Gett, A. V., and P. D. Hodgkin. 2000. A cellular calculus for signal integration by T cells. *Nat. Immunol.* 1: 239–244.
39. van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* 2: 423–429.
40. Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. *J. Immunol.* 165: 6833–6839.
41. Wong, P., and E. G. Pamer. 2001. Cutting edge: antigen-independent CD8 T cell proliferation. *J. Immunol.* 166: 5864–5868.
42. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kündig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261: 609–612.
43. Whitmire, J. K., R. A. Flavell, I. S. Grewal, C. P. Larsen, T. C. Pearson, and R. Ahmed. 1999. CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J. Immunol.* 163: 3194–3201.
44. Borrow, P., A. Tishon, S. Lee, J. Xu, I. S. Grewal, M. B. Oldstone, and R. A. Flavell. 1996. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *Journal of Experimental Medicine* 183: 2129–2142.
45. Wherry, E. J., K. A. Purro, A. Porgador, and L. C. Eisenlohr. 1999. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J.*

Immunol. 163: 3735–3745.

46. Tzelepis, F., V. Alcon, R. Dudani, K. Gurnani, A. Zafer, E. S. Everson, K. G. Young, H. Rüssmann, L. Krishnan, and S. Sad. 2012. Modulation of antigenic location converts chronic into acute infection by forcing CD8⁺ T cell recognition. *Cell Rep* 2: 1710–1721.
47. Sad, S., R. Dudani, K. Gurnani, M. Russell, H. van Faassen, B. Finlay, and L. Krishnan. 2008. Pathogen proliferation governs the magnitude but compromises the function of CD8 T cells. *J. Immunol.* 180: 5853–5861.
48. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* 15: 486–499.
49. Xiao, Z., K. A. Casey, S. C. Jameson, J. M. Curtsinger, and M. F. Mescher. 2009. Programming for CD8 T cell memory development requires IL-12 or type I IFN. *The Journal of Immunology* 182: 2786–2794.
50. Guillemins, M., F. Ginhoux, C. Jakubzick, S. H. Naik, N. Onai, B. U. Schraml, E. Segura, R. Tussiwand, and S. Yona. 2014. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* 14: 571–578.
51. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
52. Satpathy, A. T., X. Wu, J. C. Albring, and K. M. Murphy. 2012. Re(de)fining the dendritic cell lineage. *Nature Publishing Group* 13: 1145–1154.
53. Delamarre, L., M. Pack, H. Chang, I. Mellman, and E. S. Trombetta. 2005. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307: 1630–1634.
54. Accapezzato, D., V. Visco, V. Francavilla, C. Molette, T. Donato, M. Paroli, M. U. Mondelli, M. Doria, M. R. Torrisi, and V. Barnaba. 2005. Chloroquine enhances human CD8⁺ T cell responses against soluble antigens in vivo. *Journal of Experimental Medicine* 202: 817–828.
55. Savina, A., C. Jancic, S. Hugues, P. Guernonprez, P. Vargas, I. C. Moura, A.-M. Lennon-Duménil, M. C. Seabra, G. Raposo, and S. Amigorena. 2006. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126: 205–218.
56. Vulcano, M., S. Dusi, D. Lissandrini, R. Badolato, P. Mazzi, E. Riboldi, E. Borroni, A. Calleri, M. Donini, A. Plebani, L. Notarangelo, T. Musso, and S. Sozzani. 2004. Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *J. Immunol.* 173: 5749–5756.
57. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat. Immunol.* 5: 685–692.
58. Burgdorf, S., A. Kautz, V. Böhnert, P. A. Knolle, and C. Kurts. 2007. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316: 612–616.
59. Sancho, D., O. P. Joffre, A. M. Keller, N. C. Rogers, D. Martínez, P. Hernanz-Falcón, I. Rosewell, and C. R. E. Sousa. 2009. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458: 899–903.
60. Joffre, O. P., D. Sancho, S. Zelenay, A. M. Keller, and C. Reis e Sousa.

2010. Efficient and versatile manipulation of the peripheral CD4⁺ T-cell compartment by antigen targeting to DNGR-1/CLEC9A. *Eur. J. Immunol.* 40: 1255–1265.
61. Heuzé, M. L., P. Vargas, M. Chabaud, M. Le Berre, Y.-J. Liu, O. Collin, P. Solanes, R. Voituriez, M. Piel, and A.-M. Lennon-Duménil. 2013. Migration of dendritic cells: physical principles, molecular mechanisms, and functional implications. *Immunol. Rev.* 256: 240–254.
62. Auffray, C., M. H. Sieweke, and F. Geissmann. 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu. Rev. Immunol.* 27: 669–692.
63. Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26: 421–452.
64. Ginhoux, F., and S. Jung. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* 14: 392–404.
65. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8: 958–969.
66. Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S. E. W. Jacobsen, J. W. Pollard, J. Frampton, K. J. Liu, and F. Geissmann. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336: 86–90.
67. Yona, S., K.-W. Kim, Y. Wolf, A. Mildner, D. Varol, M. Breker, D. Strauss-Ayali, S. Viukov, M. Guillemins, A. Misharin, D. A. Hume, H. Perlman, B. Malissen, E. Zelzer, and S. Jung. 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38: 79–91.
68. Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov, and M. Merad. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330: 841–845.
69. Hashimoto, D., A. Chow, C. Noizat, P. Teo, M. B. Beasley, M. Leboeuf, C. D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S. W. Boyer, E. C. Forsberg, M. Tanaka, N. van Rooijen, A. García-Sastre, E. R. Stanley, F. Ginhoux, P. S. Frenette, and M. Merad. 2013. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 38: 792–804.
70. León, B., M. López-Bravo, and C. Ardavin. 2007. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against *Leishmania*. *Immunity* 26: 519–531.
71. Lavin, Y., and M. Merad. 2013. Macrophages: gatekeepers of tissue integrity. *Cancer Immunol Res* 1: 201–209.
72. Willekens, F. L. A., J. M. Werre, J. K. Kruijt, B. Roerdinkholder-Stoelwinder, Y. A. M. Groenen-Döpp, A. G. van den Bos, G. J. C. G. M. Bosman, and T. J. C. van Berkel. 2005. Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. *Blood* 105: 2141–2145.
73. Blasius, A. L., E. Giurisato, M. Cella, R. D. Schreiber, A. S. Shaw, and M.

- Colonna. 2006. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J. Immunol.* 177: 3260–3265.
74. Zhang, J., A. Raper, N. Sugita, R. Hingorani, M. Salio, M. J. Palmowski, V. Cerundolo, and P. R. Crocker. 2006. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood* 107: 3600–3608.
75. Reizis, B., M. Colonna, G. Trinchieri, F. Barrat, and M. Gilliet. 2011. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat. Rev. Immunol.* 11: 558–565.
76. Kamath, A. T., S. Henri, F. Batty, D. F. Tough, and K. Shortman. 2002. Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* 100: 1734–1741.
77. Maraskovsky, E., K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman, and H. J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *Journal of Experimental Medicine* 184: 1953–1962.
78. McKenna, H. J., K. L. Stocking, R. E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C. R. Maliszewski, D. H. Lynch, J. Smith, B. Pulendran, E. R. Roux, M. Teepe, S. D. Lyman, and J. J. Peschon. 2000. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 95: 3489–3497.
79. Waskow, C., K. Liu, G. Darrasse-Jèze, P. Guermonprez, F. Ginhoux, M. Merad, T. Shengelia, K. Yao, and M. Nussenzweig. 2008. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nature Publishing Group* 9: 676–683.
80. Bogunovic, M., F. Ginhoux, J. Helft, L. Shang, D. Hashimoto, M. Greter, K. Liu, C. Jakubzick, M. A. Ingersoll, M. Leboeuf, E. R. Stanley, M. Nussenzweig, S. A. Lira, G. J. Randolph, and M. Merad. 2009. Origin of the lamina propria dendritic cell network. *Immunity* 31: 513–525.
81. Ginhoux, F., M. P. Collin, M. Bogunovic, M. Abel, M. Leboeuf, J. Helft, J. Ochando, A. Kissenpfennig, B. Malissen, M. Grisotto, H. Snoeck, G. Randolph, and M. Merad. 2007. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J. Exp. Med.* 204: 3133–3146.
82. Haan, den, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192: 1685–1696.
83. Shortman, K., and W. R. Heath. 2010. The CD8+ dendritic cell subset. *Immunol Rev* 234: 18–31.
84. Mc Dermott, R., U. Ziylan, D. Spehner, H. Bausinger, D. Lipsker, M. Mommaas, J.-P. Cazenave, G. Raposo, B. Goud, H. de la Salle, J. Salamero, and D. Hanau. 2002. Birbeck granules are subdomains of endosomal recycling compartment in human epidermal Langerhans cells, which form where Langerin accumulates. *Mol. Biol. Cell* 13: 317–335.
85. Merad, M., F. Ginhoux, and M. Collin. 2008. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat. Rev. Immunol.* 8: 935–947.

86. Kissenpfennig, A., and B. Malissen. 2006. Langerhans cells--revisiting the paradigm using genetically engineered mice. *Trends Immunol.* 27: 132–139.
87. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18: 767–811.
88. Randolph, G. J., V. Angeli, and M. A. Swartz. 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* 5: 617–628.
89. Larregina, A. T., and L. D. Falo. 2005. Changing paradigms in cutaneous immunology: adapting with dendritic cells. *J. Invest. Dermatol.* 124: 1–12.
90. Wang, Y., K. J. Szretter, W. Vermi, S. Gilfillan, C. Rossini, M. Cella, A. D. Barrow, M. S. Diamond, and M. Colonna. 2012. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nat. Immunol.* 13: 753–760.
91. Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel, and E. G. Pamer. 2003. TNF/ iNOS -producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19: 59–70.
92. Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8 α ⁺ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol* 175: 196–200.
93. Albert, M. L., S. F. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via α v β 5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *Journal of Experimental Medicine* 188: 1359–1368.
94. Albert, M. L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392: 86–89.
95. Heath, W. R., G. T. Belz, G. M. N. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* 199: 9–26.
96. Norbury, C. C., L. J. Hewlett, A. R. Prescott, N. Shastri, and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3: 783–791.
97. Harding, C. V., and R. Song. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J. Immunol.* 153: 4925–4933.
98. Ke, Y., and J. A. Kapp. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *Journal of Experimental Medicine* 184: 1179–1184.
99. Tvinnereim, A. R., S. E. Hamilton, and J. T. Harty. 2004. Neutrophil involvement in cross-priming CD8⁺ T cell responses to bacterial antigens. *J. Immunol.* 173: 1994–2002.
100. Pozzi, L.-A. M., J. W. Maciaszek, and K. L. Rock. 2005. Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *J. Immunol.* 175: 2071–2081.

101. Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, and K. M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8 α ⁺ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–1100.
102. Desch, A. N., G. J. Randolph, K. Murphy, E. L. Gautier, R. M. Kedl, M. H. Lahoud, I. Caminschi, K. Shortman, P. M. Henson, and C. V. Jakubzick. 2011. CD103⁺ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *J. Exp. Med.* 208: 1789–1797.
103. Dalod, M., R. Chelbi, B. Malissen, and T. Lawrence. 2014. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J.* 33: 1104–1116.
104. Reis e Sousa, C., S. Hieny, T. Scharon-Kersten, D. Jankovic, H. Charest, R. N. Germain, and A. Sher. 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *Journal of Experimental Medicine* 186: 1819–1829.
105. Bachem, A., E. Hartung, S. Güttler, A. Mora, X. Zhou, A. Hegemann, M. Plantinga, E. Mazzini, P. Stoitner, S. Gurka, V. Henn, H. W. Mages, and R. A. Kroccek. 2012. Expression of XCR1 Characterizes the Batf3-Dependent Lineage of Dendritic Cells Capable of Antigen Cross-Presentation. *Front Immunol* 3: 214.
106. Edelson, B. T., W. KC, R. Juang, M. Kohyama, L. A. Benoit, P. A. Klekotka, C. Moon, J. C. Albring, W. Ise, D. G. Michael, D. Bhattacharya, T. S. Stappenbeck, M. J. Holtzman, S.-S. J. Sung, T. L. Murphy, K. Hildner, and K. M. Murphy. 2010. Peripheral CD103⁺ dendritic cells form a unified subset developmentally related to CD8 α ⁺ conventional dendritic cells. *J Exp Med* 207: 823–836.
107. Moltedo, B., W. Li, J. S. Yount, and T. M. Moran. 2011. Unique type I interferon responses determine the functional fate of migratory lung dendritic cells during influenza virus infection. *PLoS Pathog* 7: e1002345.
108. Igyártó, B. Z., K. Haley, D. Ortner, A. Bobr, M. Gerami-Nejad, B. T. Edelson, S. M. Zurawski, B. Malissen, G. Zurawski, J. Berman, and D. H. Kaplan. 2011. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 35: 260–272.
109. Mashayekhi, M., M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. 2011. CD8 α ⁺ dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35: 249–259.
110. Lawrence, T., and G. Natoli. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat. Rev. Immunol.* 11: 750–761.
111. Lorenzi, S., F. Mattei, A. Sistigu, L. Bracci, F. Spadaro, M. Sanchez, M. Spada, F. Belardelli, L. Gabriele, and G. Schiavoni. 2011. Type I IFNs control antigen retention and survival of CD8 α ⁺ dendritic cells after uptake of tumor apoptotic cells leading to cross-priming. *The Journal of Immunology* 186: 5142–5150.
112. Ahrens, S., S. Zelenay, D. Sancho, P. Hanč, S. Kjær, C. Feest, G. Fletcher, C. Durkin, A. Postigo, M. Skehel, F. Batista, B. Thompson, M. Way, C. Reis e

- Sousa, and O. Schulz. 2012. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity* 36: 635–645.
113. Albert, M. L. 2004. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat Rev Immunol* 4: 223–231.
114. Zelenay, S., and C. Reis e Sousa. 2013. Adaptive immunity after cell death. *Trends Immunol.* 34: 329–335.
115. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8⁻ dendritic cells, but cross-presented to CD8 T cells by CD8⁺ dendritic cells. *J. Immunol.* 166: 5327–5330.
116. Smith, C. M., G. T. Belz, N. S. Wilson, J. A. Villadangos, K. Shortman, F. R. Carbone, and W. R. Heath. 2003. Cutting edge: conventional CD8 alpha⁺ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *J. Immunol.* 170: 4437–4440.
117. Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting edge: conventional CD8 alpha⁺ dendritic cells are generally involved in priming CTL immunity to viruses. *J. Immunol.* 172: 1996–2000.
118. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R. M. Steinman, and K. Inaba. 2002. The CD8⁺ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *Journal of Experimental Medicine* 195: 1289–1302.
119. Allan, R. S., C. M. Smith, G. T. Belz, A. L. van Lint, L. M. Wakim, W. R. Heath, and F. R. Carbone. 2003. Epidermal viral immunity induced by CD8alpha⁺ dendritic cells but not by Langerhans cells. *Science* 301: 1925–1928.
120. Segura, E., A. L. Albiston, I. P. Wicks, S. Y. Chai, and J. A. Villadangos. 2009. Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc Natl Acad Sci USA* 106: 20377–20381.
121. Aldridge, J. R., C. E. Moseley, D. A. Boltz, N. J. Negovetich, C. Reynolds, J. Franks, S. A. Brown, P. C. Doherty, R. G. Webster, and P. G. Thomas. 2009. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 106: 5306–5311.
122. Edelson, B. T., T. R. Bradstreet, K. Hildner, J. A. Carrero, K. E. Frederick, W. KC, R. Belizaire, T. Aoshi, R. D. Schreiber, M. J. Miller, T. L. Murphy, E. R. Unanue, and K. M. Murphy. 2011. CD8α(+) dendritic cells are an obligate cellular entry point for productive infection by *Listeria monocytogenes*. *Immunity* 35: 236–248.
123. Naik, S. H., L. Perié, E. Swart, C. Gerlach, N. van Rooij, R. J. de Boer, and T. N. Schumacher. 2013. Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature* 496: 229–232.
124. Schraml, B. U., J. van Blijswijk, S. Zelenay, P. G. Whitney, A. Filby, S. E. Acton, N. C. Rogers, N. Moncaut, J. J. Carvajal, and C. Reis e Sousa. 2013. Genetic tracing via DNGR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell* 154: 843–858.
125. Satpathy, A. T., K. M. Murphy, and W. KC. 2011. Transcription factor

- networks in dendritic cell development. *Semin. Immunol.* 23: 388–397.
126. Cecchini, M. G., M. G. Dominguez, S. Mocci, A. Wetterwald, R. Felix, H. Fleisch, O. Chisholm, W. Hofstetter, J. W. Pollard, and E. R. Stanley. 1994. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 120: 1357–1372.
127. Dai, X.-M., G. R. Ryan, A. J. Hapel, M. G. Dominguez, R. G. Russell, S. Kapp, V. Sylvestre, and E. R. Stanley. 2002. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 99: 111–120.
128. Wiktor-Jedrzejczak, W., and S. Gordon. 1996. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiol. Rev.* 76: 927–947.
129. Fogg, D. K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D. R. Littman, A. Cumano, and F. Geissmann. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311: 83–87.
130. Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404: 193–197.
131. Domínguez, P. M., and C. Ardavín. 2010. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunol. Rev.* 234: 90–104.
132. Naik, S. H., P. Sathe, H.-Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J.-Y. Kwak, L. Wu, and K. Shortman. 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat. Immunol.* 8: 1217–1226.
133. Onai, N., A. Obata-Onai, M. A. Schmid, T. Ohteki, D. Jarrossay, and M. G. Manz. 2007. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat. Immunol.* 8: 1207–1216.
134. Naik, S. H., D. Metcalf, A. van Nieuwenhuijze, I. Wicks, L. Wu, M. O'Keeffe, and K. Shortman. 2006. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* 7: 663–671.
135. Liu, K., G. D. Victora, T. A. Schwickert, P. Guermonprez, M. M. Meredith, K. Yao, F.-F. Chu, G. J. Randolph, A. Y. Rudensky, and M. Nussenzweig. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science* 324: 392–397.
136. Godleski, J. J., and J. D. Brain. 1972. The origin of alveolar macrophages in mouse radiation chimeras. *Journal of Experimental Medicine* 136: 630–643.
137. Hashimoto, D., J. Miller, and M. Merad. 2011. Dendritic cell and macrophage heterogeneity in vivo. *Immunity* 35: 323–335.
138. Gautier, E. L., T. Shay, J. Miller, M. Greter, C. Jakubzick, S. Ivanov, J. Helft, A. Chow, K. G. Elpek, S. Gordonov, A. R. Mazloom, A. Ma'ayan, W.-J. Chua, T. H. Hansen, S. J. Turley, M. Merad, G. J. Randolph, Immunological Genome

- Consortium. 2012. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat. Immunol.* 13: 1118–1128.
139. Karsunky, H., M. Merad, A. Cozzio, I. L. Weissman, and M. G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3⁺ lymphoid and myeloid-committed progenitors to Flt3⁺ dendritic cells in vivo. *Journal of Experimental Medicine* 198: 305–313.
140. Maraskovsky, E., B. Pulendran, K. Brasel, M. Teepe, E. R. Roux, K. Shortman, S. D. Lyman, and H. J. McKenna. 1997. Dramatic numerical increase of functionally mature dendritic cells in FLT3 ligand-treated mice. *Adv. Exp. Med. Biol.* 417: 33–40.
141. Kingston, D., M. A. Schmid, N. Onai, A. Obata-Onai, D. Baumjohann, and M. G. Manz. 2009. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. *Blood* 114: 835–843.
142. Collin, M., N. McGovern, and M. Haniffa. 2013. Human dendritic cell subsets. *Immunology* 140: 22–30.
143. Haniffa, M., M. Collin, and F. Ginhoux. 2013. Ontogeny and functional specialization of dendritic cells in human and mouse. *Adv. Immunol.* 120: 1–49.
144. Dzionek, A., A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, D. W. Buck, and J. Schmitz. 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* 165: 6037–6046.
145. Lindstedt, M., K. Lundberg, and C. A. K. Borrebaeck. 2005. Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. *J. Immunol.* 175: 4839–4846.
146. Mcllroy, D., C. Troadec, F. Grassi, A. Samri, B. Barrou, B. Autran, P. Debré, J. Feuillard, and A. Hosmalin. 2001. Investigation of human spleen dendritic cell phenotype and distribution reveals evidence of in vivo activation in a subset of organ donors. *Blood* 97: 3470–3477.
147. Bachem, A., S. Güttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, A. Salama, K. Movassaghi, C. Opitz, H. W. Mages, V. Henn, P.-M. Kloetzel, S. Gurka, and R. A. Kroccek. 2010. Superior antigen cross-presentation and XCR1 expression define human CD11c⁺CD141⁺ cells as homologues of mouse CD8⁺ dendritic cells. *J. Exp. Med.* 207: 1273–1281.
148. Poulin, L. F., M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J.-L. Chen, A. M. Keller, O. Joffre, S. Zelenay, E. Nye, A. Le Moine, F. Faure, V. Donckier, D. Sancho, V. Cerundolo, D. Bonnet, and C. Reis e Sousa. 2010. Characterization of human DNGR-1⁺ BDCA3⁺ leukocytes as putative equivalents of mouse CD8 α ⁺ dendritic cells. *J. Exp. Med.* 207: 1261–1271.
149. Crozat, K., R. Guiton, V. Contreras, V. Feuillet, C.-A. Dutertre, E. Ventre, T.-P. Vu Manh, T. Baranek, A. K. Storset, J. Marvel, P. Boudinot, A. Hosmalin, I. Schwartz-Cornil, and M. Dalod. 2010. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8 α ⁺ dendritic cells. *J. Exp. Med.* 207: 1283–1292.
150. Haniffa, M., A. Shin, V. Bigley, N. McGovern, P. Teo, P. See, P. S. Wasan, X.-N. Wang, F. Malinarich, B. Malleret, A. Larbi, P. Tan, H. Zhao, M. Poidinger,

- S. Pagan, S. Cookson, R. Dickinson, I. Dimmick, R. F. Jarrett, L. Renia, J. Tam, C. Song, J. Connolly, J. K. Y. Chan, A. Gehring, A. Bertoletti, M. Collin, and F. Ginhoux. 2012. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity* 37: 60–73.
151. Haniffa, M., F. Ginhoux, X.-N. Wang, V. Bigley, M. Abel, I. Dimmick, S. Bullock, M. Grisotto, T. Booth, P. Taub, C. Hilkens, M. Merad, and M. Collin. 2009. Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *J. Exp. Med.* 206: 371–385.
152. Klechevsky, E., R. Morita, M. Liu, Y. Cao, S. Coquery, L. Thompson-Snipes, F. Briere, D. Chaussabel, G. Zurawski, A. K. Palucka, Y. Reiter, J. Banchereau, and H. Ueno. 2008. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity* 29: 497–510.
153. Méresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, and J. P. Gorvel. 1999. Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. *Nat. Cell Biol.* 1: E183–8.
154. Jones, B. D., and S. Falkow. 1996. Salmonellosis: host immune responses and bacterial virulence determinants. *Annu Rev Immunol* 14: 533–561.
155. Gilchrist, J. J., C. A. MacLennan, and A. V. S. Hill. 2015. Genetic susceptibility to invasive Salmonella disease. *Nat. Rev. Immunol.* 15: 452–463.
156. Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams, and A. J. Bäumlner. 2001. Animal models of Salmonella infections: enteritis versus typhoid fever. *Microbes Infect.* 3: 1335–1344.
157. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085–2088.
158. Muotiala, A., and P. H. Mäkelä. 1990. The role of IFN-gamma in murine Salmonella typhimurium infection. *Microb. Pathog.* 8: 135–141.
159. Mastroeni, P., A. Arena, G. B. Costa, M. C. Liberto, L. Bonina, and C. E. Hormaeche. 1991. Serum TNF alpha in mouse typhoid and enhancement of a Salmonella infection by anti-TNF alpha antibodies. *Microb. Pathog.* 11: 33–38.
160. Albaghdadi, H., N. Robinson, B. Finlay, L. Krishnan, and S. Sad. 2009. Selectively reduced intracellular proliferation of Salmonella enterica serovar typhimurium within APCs limits antigen presentation and development of a rapid CD8 T cell response. *J Immunol* 183: 3778–3787.
161. Luu, R. A., K. Gurnani, R. Dudani, R. Kammara, H. van Faassen, J.-C. Sirard, L. Krishnan, and S. Sad. 2006. Delayed expansion and contraction of CD8+ T cell response during infection with virulent Salmonella typhimurium. *J Immunol* 177: 1516–1525.
162. Sundquist, M., A. Rydström, and M. J. Wick. 2004. Immunity to Salmonella from a dendritic point of view. *Cell Microbiol* 6: 1–11.
163. Brennan, M. A., and B. T. Cookson. 2000. Salmonella induces macrophage death by caspase-1-dependent necrosis. *Mol. Microbiol.* 38: 31–40.
164. Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow. 1996.

- Salmonella typhimurium invasion induces apoptosis in infected macrophages. *Proceedings of the National Academy of Sciences* 93: 9833–9838.
165. Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay. 2000. Salmonella pathogenicity islands: big virulence in small packages. *Microbes Infect.* 2: 145–156.
166. Haraga, A., M. B. Ohlson, and S. I. Miller. 2008. Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* 6: 53–66.
167. Salcedo, S. P., M. Noursadeghi, J. Cohen, and D. W. Holden. 2001. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. *Cell. Microbiol.* 3: 587–597.
168. Galán, J. E. 2001. Salmonella interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* 17: 53–86.
169. Leung, K. Y., and B. B. Finlay. 1991. Intracellular replication is essential for the virulence of Salmonella typhimurium. *Proc. Natl. Acad. Sci. U.S.A.* 88: 11470–11474.
170. Robson, H. G., and S. I. Vas. 1972. Resistance of inbred mice to Salmonella typhimurium. *J. Infect. Dis.* 126: 378–386.
171. Arpaia, N., J. Godec, L. Lau, K. E. Sivick, L. M. McLaughlin, M. B. Jones, T. Dracheva, S. N. Peterson, D. M. Monack, and G. M. Barton. 2011. TLR signaling is required for Salmonella typhimurium virulence. *Cell* 144: 675–688.
172. Sivick, K. E., N. Arpaia, G. L. Reiner, B. L. Lee, B. R. Russell, and G. M. Barton. 2014. Toll-like receptor-deficient mice reveal how innate immune signaling influences Salmonella virulence strategies. *Cell Host Microbe* 15: 203–213.
173. Arpaia, N., and G. M. Barton. 2013. The impact of Toll-like receptors on bacterial virulence strategies. *Curr. Opin. Microbiol.* 16: 17–22.
174. Franchi, L., N. Warner, K. Viani, and G. Nuñez. 2009. Function of Nod-like receptors in microbial recognition and host defense. *Immunol. Rev.* 227: 106–128.
175. Raupach, B., S.-K. Peuschel, D. M. Monack, and A. Zychlinsky. 2006. Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against Salmonella enterica serovar Typhimurium infection. *Infect. Immun.* 74: 4922–4926.
176. Broz, P., K. Newton, M. Lamkanfi, S. Mariathasan, V. M. Dixit, and D. M. Monack. 2010. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J. Exp. Med.* 207: 1745–1755.
177. Zhao, Y., and F. Shao. 2015. The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. *Immunol. Rev.* 265: 85–102.
178. Vidal, S. M., E. Pinner, P. Lepage, S. Gauthier, and P. Gros. 1996. Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains. *J Immunol* 157: 3559–3568.
179. Vidal, S. M., D. Malo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* 73: 469–485.

180. Govoni, G., S. Vidal, S. Gauthier, E. Skamene, D. Malo, and P. Gros. 1996. The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57BL/6J mice transgenic for the Nramp1 Gly169 allele. *Infect. Immun.* 64: 2923–2929.
181. Forbes, J. R., and P. Gros. 2001. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol.* 9: 397–403.
182. Fritsche, G., M. Nairz, S. J. Libby, F. C. Fang, and G. Weiss. 2012. Slc11a1 (Nramp1) impairs growth of *Salmonella enterica* serovar typhimurium in macrophages via stimulation of lipocalin-2 expression. *J. Leukoc. Biol.* 92: 353–359.
183. Vidal, S., M. L. Tremblay, G. Govoni, S. Gauthier, G. Sebastiani, D. Malo, E. Skamene, M. Olivier, S. Jothy, and P. Gros. 1995. The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *Journal of Experimental Medicine* 182: 655–666.
184. Sancho-Shimizu, V., R. Khan, S. Mostowy, L. Larivière, R. Wilkinson, N. Riendeau, M. Behr, and D. Malo. 2007. Molecular genetic analysis of two loci (Ity2 and Ity3) involved in the host response to infection with *Salmonella typhimurium* using congenic mice and expression profiling. *Genetics* 177: 1125–1139.
185. Roy, M.-F., and D. Malo. 2002. Genetic regulation of host responses to *Salmonella* infection in mice. *Genes Immun.* 3: 381–393.
186. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc. R. Soc. Lond., B, Biol. Sci.* 147: 258–267.
187. Thomas, C., I. Moraga, D. Levin, P. O. Krutzik, Y. Podoplelova, A. Trejo, C. Lee, G. Yarden, S. E. Vleck, J. S. Glenn, G. P. Nolan, J. Piehler, G. Schreiber, and K. C. Garcia. 2011. Structural linkage between ligand discrimination and receptor activation by type I interferons. *Cell* 146: 621–632.
188. Sadler, A. J., and B. R. G. Williams. 2008. Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* 8: 559–568.
189. Megjugorac, N. J., G. E. Gallagher, and G. Gallagher. 2009. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *J. Leukoc. Biol.* 86: 1359–1363.
190. McNab, F., K. Mayer-Barber, A. Sher, A. Wack, and A. O'Garra. 2015. Type I interferons in infectious disease. *Nat. Rev. Immunol.* 15: 87–103.
191. Colonna, M., G. Trinchieri, and Y.-J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–1226.
192. Swiecki, M., S. Gilfillan, W. Vermi, Y. Wang, and M. Colonna. 2010. Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* 33: 955–966.
193. Crouse, J., U. Kalinke, and A. Oxenius. 2015. Regulation of antiviral T cell responses by type I interferons. *Nat. Rev. Immunol.* 15: 231–242.
194. Hahm, B., M. J. Trifilo, E. I. Zuniga, and M. B. A. Oldstone. 2005. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity* 22: 247–257.
195. Tamura, T., P. Tailor, K. Yamaoka, H. J. Kong, H. Tsujimura, J. J. O'Shea, H. Singh, and K. Ozato. 2005. IFN regulatory factor-4 and -8 govern dendritic cell

subset development and their functional diversity. *J. Immunol.* 174: 2573–2581.

196. Paquette, R. L., N. C. Hsu, S. M. Kiertscher, A. N. Park, L. Tran, M. D. Roth, and J. A. Glaspy. 1998. Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells. *J. Leukoc. Biol.* 64: 358–367.

197. Santini, S. M., C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. 2000. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *Journal of Experimental Medicine* 191: 1777–1788.

198. Parlato, S., S. M. Santini, C. Lapenta, T. Di Pucchio, M. Logozzi, M. Spada, A. M. Giammarioli, W. Malorni, S. Fais, and F. Belardelli. 2001. Expression of CCR-7, MIP-3beta, and Th-1 chemokines in type I IFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities. *Blood* 98: 3022–3029.

199. Rouzaut, A., S. Garasa, A. Teijeira, I. González, I. Martinez-Forero, N. Suarez, E. Larrea, C. Alfaro, A. Palazón, J. Dubrot, S. Hervás-Stubbs, and I. Melero. 2010. Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN- α . *Eur. J. Immunol.* 40: 3054–3063.

200. Cousens, L. P., J. S. Orange, H. C. Su, and C. A. Biron. 1997. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. *Proceedings of the National Academy of Sciences* 94: 634–639.

201. Byrnes, A. A., X. Ma, P. Cuomo, K. Park, L. Wahl, S. F. Wolf, H. Zhou, G. Trinchieri, and C. L. Karp. 2001. Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. *Eur. J. Immunol.* 31: 2026–2034.

202. Padovan, E., G. C. Spagnoli, M. Ferrantini, and M. Heberer. 2002. IFN-alpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8+ effector T cells. *J. Leukoc. Biol.* 71: 669–676.

203. Brierley, M. M., and E. N. Fish. 2005. Stats: multifaceted regulators of transcription. *J. Interferon Cytokine Res.* 25: 733–744.

204. Plataniias, L. C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* 5: 375–386.

205. Tanabe, Y., T. Nishibori, L. Su, R. M. Arduini, D. P. Baker, and M. David. 2005. Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. *J. Immunol.* 174: 609–613.

206. Gimeno, R., C.-K. Lee, C. Schindler, and D. E. Levy. 2005. Stat1 and Stat2 but not Stat3 arbitrate contradictory growth signals elicited by alpha/beta interferon in T lymphocytes. *Mol. Cell. Biol.* 25: 5456–5465.

207. Simmons, D. P., P. A. Wearsch, D. H. Canaday, H. J. Meyerson, Y. C. Liu, Y. Wang, W. H. Boom, and C. V. Harding. 2012. Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. *The Journal of Immunology* 188: 3116–3126.

208. Le Bon, A., and D. F. Tough. 2008. Type I interferon as a stimulus for cross-priming. *Cytokine Growth Factor Rev* 19: 33–40.

209. Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D. F. Tough. 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99: 3263–3271.
210. Wilson, E. B., Y. Kidani, H. Elsaesser, J. Barnard, L. Raff, C. L. Karp, S. Bensinger, and D. G. Brooks. 2012. Emergence of distinct multiarmed immunoregulatory antigen-presenting cells during persistent viral infection. *Cell Host Microbe* 11: 481–491.
211. Honke, N., N. Shaabani, G. Cadeddu, U. R. Sorg, D.-E. Zhang, M. Trilling, K. Klingel, M. Sauter, R. Kandolf, N. Gailus, N. van Rooijen, C. Burkart, S. E. Baldus, M. Grusdat, M. Löhning, H. Hengel, K. Pfeffer, M. Tanaka, D. Häussinger, M. Recher, P. A. Lang, and K. S. Lang. 2012. Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. *Nat Immunol* 13: 51–57.
212. Nierkens, S., J. Tel, E. Janssen, and G. J. Adema. 2013. Antigen cross-presentation by dendritic cell subsets: one general or all sergeants? *Trends Immunol.* 34: 361–370.
213. Le Bon, A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol* 4: 1009–1015.
214. Spadaro, F., C. Lapenta, S. Donati, L. Abalsamo, V. Barnaba, F. Belardelli, S. M. Santini, and M. Ferrantini. 2012. IFN- α enhances cross-presentation in human dendritic cells by modulating antigen survival, endocytic routing, and processing. *Blood* 119: 1407–1417.
215. Savina, A., A. Peres, I. Cebrian, N. Carmo, C. Moita, N. Hacohen, L. F. Moita, and S. Amigorena. 2009. The small GTPase Rac2 controls phagosomal alkalization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 30: 544–555.
216. Pasparakis, M., and P. Vandenabeele. 2015. Necroptosis and its role in inflammation. *Nature* 517: 311–320.
217. Vandenabeele, P., L. Galluzzi, T. Vanden Berghe, and G. Kroemer. 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 11: 700–714.
218. Kerr, J. F., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26: 239–257.
219. McIlwain, D. R., T. Berger, and T. W. Mak. 2013. Caspase Functions in Cell Death and Disease. *Cold Spring Harbor Perspectives in Biology* 5: a008656–a008656.
220. Yrlid, U., and M. J. Wick. 2000. Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. *J Exp Med* 191: 613–624.
221. Bosnjak, L., M. Miranda-Saksena, D. M. Koelle, R. A. Boadle, C. A. Jones, and A. L. Cunningham. 2005. Herpes simplex virus infection of human dendritic cells induces apoptosis and allows cross-presentation via uninfected dendritic cells. *J. Immunol.* 174: 2220–2227.
222. Blander, J. M. 2014. A long-awaited merger of the pathways mediating host

- defence and programmed cell death. *Nature Publishing Group* 14: 601–618.
223. Majno, G., and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *The American Journal of Pathology* 146: 3–15.
224. Broz, P., and D. M. Monack. 2011. Molecular mechanisms of inflammasome activation during microbial infections. *Immunol. Rev.* 243: 174–190.
225. Fink, S. L., and B. T. Cookson. 2007. Pyroptosis and host cell death responses during *Salmonella* infection. *Cell Microbiol* 9: 2562–2570.
226. Thapa, R. J., S. Nogusa, P. Chen, J. L. Maki, A. Lerro, M. Andrade, G. F. Rall, A. Degterev, and S. Balachandran. 2013. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. *Proc. Natl. Acad. Sci. U.S.A.* 110: E3109–18.
227. Degterev, A., Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G. D. Cuny, T. J. Mitchison, M. A. Moskowitz, and J. Yuan. 2005. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat. Chem. Biol.* 1: 112–119.
228. Cho, Y. S., S. Challa, D. Moquin, R. Genga, T. D. Ray, M. Guildford, and F. K.-M. Chan. 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137: 1112–1123.
229. Christofferson, D. E., and J. Yuan. 2010. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol* 22: 263–268.
230. Declercq, W., T. Vanden Berghe, and P. Vandenabeele. 2009. RIP Kinases at the Crossroads of Cell Death and Survival. *Cell* 138: 229–232.
231. Robinson, N., S. McComb, R. Mulligan, R. Dudani, L. Krishnan, and S. Sad. 2012. Type I interferon induces necroptosis in macrophages during infection with *Salmonella enterica* serovar Typhimurium. *Nat. Immunol.* 13: 954–962.
232. Li, J., T. McQuade, A. B. Siemer, J. Napetschnig, K. Moriwaki, Y.-S. Hsiao, E. Damko, D. Moquin, T. Walz, A. McDermott, F. K.-M. Chan, and H. Wu. 2012. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* 150: 339–350.
233. Murphy, J. M., P. E. Czabotar, J. M. Hildebrand, I. S. Lucet, J.-G. Zhang, S. Alvarez-Diaz, R. Lewis, N. Lalaoui, D. Metcalf, A. I. Webb, S. N. Young, L. N. Varghese, G. M. Tannahill, E. C. Hatchell, I. J. Majewski, T. Okamoto, R. C. J. Dobson, D. J. Hilton, J. J. Babon, N. A. Nicola, A. Strasser, J. Silke, and W. S. Alexander. 2013. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* 39: 443–453.
234. Dondelinger, Y., W. Declercq, S. Montessuit, R. Roelandt, A. Goncalves, I. Bruggeman, P. Hulpiau, K. Weber, C. A. Sehon, R. W. Marquis, J. Bertin, P. J. Gough, S. Savvides, J.-C. Martinou, M. J. M. Bertrand, and P. Vandenabeele. 2014. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep* 7: 971–981.
235. Wang, H., L. Sun, L. Su, J. Rizo, L. Liu, L.-F. Wang, F.-S. Wang, and X. Wang. 2014. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Molecular Cell* 54: 133–146.
236. Moriwaki, K., and F. K. M. Chan. 2013. RIP3: a molecular switch for

necrosis and inflammation. *Genes & Development* 27: 1640–1649.

237. Moriwaki, K., J. Bertin, P. J. Gough, and F. K. M. Chan. 2015. A RIPK3-Caspase 8 Complex Mediates Atypical Pro-IL-1 Processing. *J. Immunol.* 194: 1938–1944.

238. Lamkanfi, M., and V. M. Dixit. 2014. Mechanisms and functions of inflammasomes. *Cell* 157: 1013–1022.

239. Groß, O., C. J. Thomas, G. Guarda, and J. Tschopp. 2011. The inflammasome: an integrated view. *Immunol. Rev.* 243: 136–151.

240. Lopez-Castejon, G., and D. Brough. 2011. Understanding the mechanism of IL-1 β secretion. *Cytokine Growth Factor Rev.* 22: 189–195.

241. Monie, T. P., and C. E. Bryant. 2015. Caspase-8 functions as a key mediator of inflammation and pro-IL-1 β processing via both canonical and non-canonical pathways. *Immunol. Rev.* 265: 181–193.

242. Sulston, J. E., and H. R. Horvitz. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* 56: 110–156.

243. Meier, P., A. Finch, and G. Evan. 2000. Apoptosis in development. *Nature* 407: 796–801.

244. Donepudi, M., and M. G. Grütter. 2002. Structure and zymogen activation of caspases. *Biophys. Chem.* 101-102: 145–153.

245. Boatright, K. M., and G. S. Salvesen. 2003. Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* 15: 725–731.

246. Scott, A. M., and M. Saleh. 2007. The inflammatory caspases: guardians against infections and sepsis. *Cell Death Differ.* 14: 23–31.

247. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular Cell* 10: 417–426.

248. Jang, T.-H., C. Zheng, J. Li, C. Richards, Y.-S. Hsiao, T. Walz, H. Wu, and H. H. Park. 2014. Structural study of the RIPoptosome core reveals a helical assembly for kinase recruitment. *Biochemistry* 53: 5424–5431.

249. Stennicke, H. R., J. M. Jürgensmeier, H. Shin, Q. Deveraux, B. B. Wolf, X. Yang, Q. Zhou, H. M. Ellerby, L. M. Ellerby, D. Bredesen, D. R. Green, J. C. Reed, C. J. Froelich, and G. S. Salvesen. 1998. Pro-caspase-3 is a major physiologic target of caspase-8. *J. Biol. Chem.* 273: 27084–27090.

250. Tait, S. W. G., and D. R. Green. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat. Rev. Mol. Cell Biol.* 11: 621–632.

251. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132–1136.

252. Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275: 1129–1132.

253. Riedl, S. J., and G. S. Salvesen. 2007. The apoptosome: signalling platform of cell death. *Nat. Rev. Mol. Cell Biol.* 8: 405–413.

254. Vercammen, D., R. Beyaert, G. Denecker, V. Goossens, G. Van Loo, W. Declercq, J. Grooten, W. Fiers, and P. Vandenabeele. 1998. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor

- necrosis factor. *Journal of Experimental Medicine* 187: 1477–1485.
255. Mocarski, E. S., J. W. Upton, and W. J. Kaiser. 2012. Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. *Nat. Rev. Immunol.* 12: 79–88.
256. McComb, S., H. H. Cheung, R. G. Korneluk, S. Wang, L. Krishnan, and S. Sad. 2012. cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. *Cell Death Differ.* 19: 1791–1801.
257. Oberst, A., C. P. Dillon, R. Weinlich, L. L. McCormick, P. Fitzgerald, C. Pop, R. Hakem, G. S. Salvesen, and D. R. Green. 2011. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 471: 363–367.
258. Kaiser, W. J., J. W. Upton, A. B. Long, D. Livingston-Rosanoff, L. P. Daley-Bauer, R. Hakem, T. Caspary, and E. S. Mocarski. 2011. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 471: 368–372.
259. Callus, B. A., and D. L. Vaux. 2007. Caspase inhibitors: viral, cellular and chemical. *Cell Death Differ.* 14: 73–78.
260. Mack, C., A. Sickmann, D. Lembo, and W. Brune. 2008. Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. *Proc. Natl. Acad. Sci. U.S.A.* 105: 3094–3099.
261. Upton, J. W., W. J. Kaiser, and E. S. Mocarski. 2008. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. *J. Biol. Chem.* 283: 16966–16970.
262. Upton, J. W., W. J. Kaiser, and E. S. Mocarski. 2012. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host Microbe* 11: 290–297.
263. Upton, J. W., W. J. Kaiser, and E. S. Mocarski. 2010. Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe* 7: 302–313.
264. Kaczmarek, A., P. Vandenabeele, and D. V. Krysko. 2013. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 38: 209–223.
265. Micheau, O., and J. Tschopp. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114: 181–190.
266. Feoktistova, M., P. Geserick, B. Kellert, D. P. Dimitrova, C. Langlais, M. Hupe, K. Cain, M. MacFarlane, G. Häcker, and M. Leverkus. 2011. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Molecular Cell* 43: 449–463.
267. Tenev, T., K. Bianchi, M. Darding, M. Broemer, C. Langlais, F. Wallberg, A. Zachariou, J. Lopez, M. MacFarlane, K. Cain, and P. Meier. 2011. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Molecular Cell* 43: 432–448.
268. Brenner, D., H. Blaser, and T. W. Mak. 2015. Regulation of tumour necrosis factor signalling: live or let die. *Nat. Rev. Immunol.* 15: 362–374.
269. Silke, J., J. A. Rickard, and M. Gerlic. 2015. The diverse role of RIP kinases in necroptosis and inflammation. *Nature Publishing Group* 16: 689–697.
270. Khan, N., K. E. Lawlor, J. M. Murphy, and J. E. Vince. 2014. More to life

than death: molecular determinants of necroptotic and non-necroptotic RIP3 kinase signaling. *Curr. Opin. Immunol.* 26: 76–89.

271. Wu, X.-N., Z.-H. Yang, X.-K. Wang, Y. Zhang, H. Wan, Y. Song, X. Chen, J. Shao, and J. Han. 2014. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. *Cell Death Differ.* 21: 1709–1720.

272. Degterev, A., J. Hitomi, M. Germscheid, I. L. Ch'en, O. Korkina, X. Teng, D. Abbott, G. D. Cuny, C. Yuan, G. Wagner, S. M. Hedrick, S. A. Gerber, A. Lugovskoy, and J. Yuan. 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* 4: 313–321.

273. Orozco, S., N. Yatim, M. R. Werner, H. Tran, S. Y. Gunja, S. W. G. Tait, M. L. Albert, D. R. Green, and A. Oberst. 2014. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death Differ.* 21: 1511–1521.

274. Sun, L., H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei, and X. Wang. 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148: 213–227.

275. Zhao, J., S. Jitkaew, Z. Cai, S. Choksi, Q. Li, J. Luo, and Z.-G. Liu. 2012. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc. Natl. Acad. Sci. U.S.A.* 109: 5322–5327.

276. Cai, Z., S. Jitkaew, J. Zhao, H.-C. Chiang, S. Choksi, J. Liu, Y. Ward, L.-G. Wu, and Z.-G. Liu. 2014. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat. Cell Biol.* 16: 55–65.

277. Chen, X., W. Li, J. Ren, D. Huang, W.-T. He, Y. Song, C. Yang, W. Li, X. Zheng, P. Chen, and J. Han. 2014. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. *Cell Res.* 24: 105–121.

278. Wang, Z., H. Jiang, S. Chen, F. Du, and X. Wang. 2012. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 148: 228–243.

279. Zhao, Y., X. Sui, and H. Ren. 2010. From procaspase-8 to caspase-8: revisiting structural functions of caspase-8. *J. Cell. Physiol.* 225: 316–320.

280. Hughes, M. A., N. Harper, M. Butterworth, K. Cain, G. M. Cohen, and M. MacFarlane. 2009. Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. *Molecular Cell* 35: 265–279.

281. Oberst, A., C. Pop, A. G. Tremblay, V. Blais, J.-B. Denault, G. S. Salvesen, and D. R. Green. 2010. Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *J. Biol. Chem.* 285: 16632–16642.

282. Afonina, I. S., C. Müller, S. J. Martin, and R. Beyaert. 2015. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity* 42: 991–1004.

283. Hazuda, D. J., J. Strickler, F. Kueppers, P. L. Simon, and P. R. Young. 1990. Processing of precursor interleukin 1 beta and inflammatory disease. *J. Biol. Chem.* 265: 6318–6322.

284. Korkmaz, B., M. S. Horwitz, D. E. Jenne, and F. Gauthier. 2010. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol. Rev.* 62: 726–759.
285. Maelfait, J., E. Vercammen, S. Janssens, P. Schotte, M. Haegman, S. Magez, and R. Beyaert. 2008. Stimulation of Toll-like receptor 3 and 4 induces interleukin-1 maturation by caspase-8. *Journal of Experimental Medicine* 205: 1967–1973.
286. Antonopoulos, C., C. El Sanadi, W. J. Kaiser, E. S. Mocarski, and G. R. Dubyak. 2013. Proapoptotic chemotherapeutic drugs induce noncanonical processing and release of IL-1 β via caspase-8 in dendritic cells. *The Journal of Immunology* 191: 4789–4803.
287. Varfolomeev, E. E., M. Schuchmann, V. Luria, N. Chiannikulchai, J. S. Beckmann, I. L. Mett, D. Rebrikov, V. M. Brodianski, O. C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K. B. Avraham, T. Goncharov, H. Holtmann, P. Lonai, and D. Wallach. 1998. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 9: 267–276.
288. Yeh, W. C., J. L. de la Pompa, M. E. McCurrach, H. B. Shu, A. J. Elia, A. Shahinian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, W. S. El-Deiry, S. W. Lowe, D. V. Goeddel, and T. W. Mak. 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* 279: 1954–1958.
289. Yeh, W. C., A. Itie, A. J. Elia, M. Ng, H. B. Shu, A. Wakeham, C. Mirtsos, N. Suzuki, M. Bonnard, D. V. Goeddel, and T. W. Mak. 2000. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* 12: 633–642.
290. O'Donnell, M. A., E. Perez-Jimenez, A. Oberst, A. Ng, R. Massoumi, R. Xavier, D. R. Green, and A. T. Ting. 2011. Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat. Cell Biol.* 13: 1437–1442.
291. Lopez, J., and P. Meier. 2010. To fight or die - inhibitor of apoptosis proteins at the crossroad of innate immunity and death. *Curr. Opin. Cell Biol.* 22: 872–881.
292. O'Riordan, M. X. D., L. D. Bauler, F. L. Scott, and C. S. Duckett. 2008. Inhibitor of apoptosis proteins in eukaryotic evolution and development: a model of thematic conservation. *Dev. Cell* 15: 497–508.
293. Hunter, A. M., E. C. LaCasse, and R. G. Korneluk. 2007. The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis* 12: 1543–1568.
294. Eckelman, B. P., G. S. Salvesen, and F. L. Scott. 2006. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep.* 7: 988–994.
295. Eckelman, B. P., and G. S. Salvesen. 2006. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J. Biol. Chem.* 281: 3254–3260.
296. Gyrd-Hansen, M., and P. Meier. 2010. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nature Publishing Group* 10: 561–574.

297. Lorick, K. L., J. P. Jensen, S. Fang, A. M. Ong, S. Hatakeyama, and A. M. Weissman. 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proceedings of the National Academy of Sciences* 96: 11364–11369.
298. Varfolomeev, E., J. W. Blankenship, S. M. Wayson, A. V. Fedorova, N. Kayagaki, P. Garg, K. Zobel, J. N. Dynek, L. O. Elliott, H. J. A. Wallweber, J. A. Flygare, W. J. Fairbrother, K. Deshayes, V. M. Dixit, and D. Vucic. 2007. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 131: 669–681.
299. Vince, J. E., W. W.-L. Wong, N. Khan, R. Feltham, D. Chau, A. U. Ahmed, C. A. Benetatos, S. K. Chundururu, S. M. Condon, M. McKinlay, R. Brink, M. Leverkus, V. Tergaonkar, P. Schneider, B. A. Callus, F. Koentgen, D. L. Vaux, and J. Silke. 2007. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 131: 682–693.
300. Ea, C.-K., L. Deng, Z.-P. Xia, G. Pineda, and Z. J. Chen. 2006. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Molecular Cell* 22: 245–257.
301. Wu, C.-J., D. B. Conze, T. Li, S. M. Srinivasula, and J. D. Ashwell. 2006. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. *Nat. Cell Biol.* 8: 398–406.
302. Oeckinghaus, A., M. S. Hayden, and S. Ghosh. 2011. Crosstalk in NF-kB signaling pathways. *Nat. Immunol.* 12: 695–708.
303. Zobel, K., L. Wang, E. Varfolomeev, M. C. Franklin, L. O. Elliott, H. J. A. Wallweber, D. C. Okawa, J. A. Flygare, D. Vucic, W. J. Fairbrother, and K. Deshayes. 2006. Design, synthesis, and biological activity of a potent Smac mimetic that sensitizes cancer cells to apoptosis by antagonizing IAPs. *ACS Chem. Biol.* 1: 525–533.
304. Mahoney, D. J., H. H. Cheung, R. L. Mrad, S. Plenchette, C. Simard, E. Enwere, V. Arora, T. W. Mak, E. C. Lacasse, J. Waring, and R. G. Korneluk. 2008. Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. *Proc. Natl. Acad. Sci. U.S.A.* 105: 11778–11783.
305. Stanger, B. Z., P. Leder, T. H. Lee, E. Kim, and B. Seed. 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 81: 513–523.
306. Park, H. H., Y.-C. Lo, S.-C. Lin, L. Wang, J. K. Yang, and H. Wu. 2007. The death domain superfamily in intracellular signaling of apoptosis and inflammation. *Annu. Rev. Immunol.* 25: 561–586.
307. Zhang, D., J. Lin, and J. Han. 2010. Receptor-interacting protein (RIP) kinase family. *Cell. Mol. Immunol.* 7: 243–249.
308. Humphries, F., S. Yang, B. Wang, and P. N. Moynagh. 2015. RIP kinases: key decision makers in cell death and innate immunity. *Cell Death Differ.* 22: 225–236.
309. Vandenabeele, P., W. Declercq, F. Van Herreweghe, and T. Vanden Berghe. 2010. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Sci Signal* 3: re4–re4.
310. Vandenabeele, P., S. Grootjans, N. Callewaert, and N. Takahashi. 2013.

Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. *Cell Death Differ.* 20: 185–187.

311. Rickard, J. A., J. A. O'Donnell, J. M. Evans, N. Lalaoui, A. R. Poh, T. Rogers, J. E. Vince, K. E. Lawlor, R. L. Ninnis, H. Anderton, C. Hall, S. K. Spall, T. J. Pheesse, H. E. Abud, L. H. Cengia, J. Corbin, S. Mifsud, L. Di Rago, D. Metcalf, M. Ernst, G. Dewson, A. W. Roberts, W. S. Alexander, J. M. Murphy, P. G. Ekert, S. L. Masters, D. L. Vaux, B. A. Croker, M. Gerlic, and J. Silke. 2014. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell* 157: 1175–1188.

312. Kaiser, W. J., L. P. Daley-Bauer, R. J. Thapa, P. Mandal, S. B. Berger, C. Huang, A. Sundararajan, H. Guo, L. Roback, S. H. Speck, J. Bertin, P. J. Gough, S. Balachandran, and E. S. Mocarski. 2014. RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. *Proc. Natl. Acad. Sci. U.S.A.* 111: 7753–7758.

313. Polykratis, A., N. Hermance, M. Zelic, J. Roderick, C. Kim, T.-M. Van, T. H. Lee, F. K. M. Chan, M. Pasparakis, and M. A. Kelliher. 2014. Cutting edge: RIPK1 Kinase inactive mice are viable and protected from TNF-induced necroptosis in vivo. *The Journal of Immunology* 193: 1539–1543.

314. Berger, S. B., V. Kasparcova, S. Hoffman, B. Swift, L. Dare, M. Schaeffer, C. Capriotti, M. Cook, J. Finger, A. Hughes-Earle, P. A. Harris, W. J. Kaiser, E. S. Mocarski, J. Bertin, and P. J. Gough. 2014. Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *The Journal of Immunology* 192: 5476–5480.

315. Newton, K., D. L. Dugger, K. E. Wickliffe, N. Kapoor, M. C. de Almagro, D. Vucic, L. Kömüves, R. E. Ferrando, D. M. French, J. Webster, M. Roose-Girma, S. Warming, and V. M. Dixit. 2014. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. *Science* 343: 1357–1360.

316. Newton, K., X. Sun, and V. M. Dixit. 2004. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 24: 1464–1469.

317. Christofferson, D. E., Y. Li, and J. Yuan. 2014. Control of life-or-death decisions by RIP1 kinase. *Annu. Rev. Physiol.* 76: 129–150.

318. Lawlor, K. E., N. Khan, A. Mildenhall, M. Gerlic, B. A. Croker, A. A. D'Cruz, C. Hall, S. Kaur Spall, H. Anderton, S. L. Masters, M. Rashidi, I. P. Wicks, W. S. Alexander, Y. Mitsuuchi, C. A. Benetatos, S. M. Condon, W. W.-L. Wong, J. Silke, D. L. Vaux, and J. E. Vince. 2015. RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nature Communications* 6: 6282–19.

319. Shi, J., Y. Zhao, Y. Wang, W. Gao, J. Ding, P. Li, L. Hu, and F. Shao. 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514: 187–192.

320. Bergsbaken, T., S. L. Fink, and B. T. Cookson. 2009. Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7: 99–109.

321. Stutz, A., G. L. Horvath, B. G. Monks, and E. Latz. 2013. ASC speck formation as a readout for inflammasome activation. *Methods Mol. Biol.* 1040: 91–101.

322. Qu, Y., S. Misaghi, A. Izrael-Tomasevic, K. Newton, L. L. Gilmour, M. Lamkanfi, S. Louie, N. Kayagaki, J. Liu, L. Kömüves, J. E. Cupp, D. Arnott, D. Monack, and V. M. Dixit. 2012. Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature* 490: 539–542.
323. Juliana, C., T. Fernandes-Alnemri, S. Kang, A. Farias, F. Qin, and E. S. Alnemri. 2012. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J. Biol. Chem.* 287: 36617–36622.
324. Py, B. F., M.-S. Kim, H. Vakifahmetoglu-Norberg, and J. Yuan. 2013. Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Molecular Cell* 49: 331–338.
325. Miao, E. A., J. V. Rajan, and A. Aderem. 2011. Caspase-1-induced pyroptotic cell death. *Immunol. Rev.* 243: 206–214.
326. Fernandes-Alnemri, T., J. Wu, J.-W. Yu, P. Datta, B. Miller, W. Jankowski, S. Rosenberg, J. Zhang, and E. S. Alnemri. 2007. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ.* 14: 1590–1604.
327. Proell, M., M. Gerlic, P. D. Mace, J. C. Reed, and S. J. Riedl. 2013. The CARD plays a critical role in ASC foci formation and inflammasome signalling. *Biochem. J.* 449: 613–621.
328. Antonopoulos, C., H. M. Russo, C. El Sanadi, B. N. Martin, X. Li, W. J. Kaiser, E. S. Mocarski, and G. R. Dubyak. 2015. Caspase-8 as an Effector and Regulator of NLRP3 Inflammasome Signaling. *J. Biol. Chem.* jbc.M115.652321–33.
329. Wallach, D., T.-B. Kang, and A. Kovalenko. 2014. Concepts of tissue injury and cell death in inflammation: a historical perspective. *Nat. Rev. Immunol.* 14: 51–59.
330. Dinarello, C. A., and J. W. M. van der Meer. 2013. Treating inflammation by blocking interleukin-1 in humans. *Semin. Immunol.* 25: 469–484.
331. Dinarello, C. A. 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 117: 3720–3732.
332. Broz, P., J. von Moltke, J. W. Jones, R. E. Vance, and D. M. Monack. 2010. Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* 8: 471–483.
333. Miao, E. A., I. A. Leaf, P. M. Treuting, D. P. Mao, M. Dors, A. Sarkar, S. E. Warren, M. D. Wewers, and A. Aderem. 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nature Publishing Group* 11: 1136–1142.
334. Garlanda, C., C. A. Dinarello, and A. Mantovani. 2013. The interleukin-1 family: back to the future. *Immunity* 39: 1003–1018.
335. Carruth, L. M., S. Demczuk, and S. B. Mizel. 1991. Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. *J. Biol. Chem.* 266: 12162–12167.
336. Kobayashi, Y., K. Yamamoto, T. Saido, H. Kawasaki, J. J. Oppenheim, and K. Matsushima. 1990. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. *Proceedings of the National Academy of Sciences* 87: 5548–5552.

337. Afonina, I. S., G. A. Tynan, S. E. Logue, S. P. Cullen, M. Bots, A. U. Lüthi, E. P. Reeves, N. G. McElvaney, J. P. Medema, E. C. Lavelle, and S. J. Martin. 2011. Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1 α . *Molecular Cell* 44: 265–278.
338. Hiscott, J., J. Marois, J. Garoufalis, M. D'Addario, A. Roulston, I. Kwan, N. Pepin, J. Lacoste, H. Nguyen, and G. Bensi. 1993. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol. Cell. Biol.* 13: 6231–6240.
339. Cogswell, J. P., M. M. Godlevski, G. B. Wisely, W. C. Clay, L. M. Leesnitzer, J. P. Ways, and J. G. Gray. 1994. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J. Immunol.* 153: 712–723.
340. Guma, M., L. Ronacher, R. Liu-Bryan, S. Takai, M. Karin, and M. Corr. 2009. Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis Rheum.* 60: 3642–3650.
341. Parnet, P., K. E. Garka, T. P. Bonnert, S. K. Dower, and J. E. Sims. 1996. IL-1Rrp is a novel receptor-like molecule similar to the type I interleukin-1 receptor and its homologues T1/ST2 and IL-1R AcP. *J. Biol. Chem.* 271: 3967–3970.
342. Dinarello, C. A. 1999. Interleukin-18. *Methods* 19: 121–132.
343. Fantuzzi, G., and C. A. Dinarello. 1999. Interleukin-18 and interleukin-1 beta: two cytokine substrates for ICE (caspase-1). *J. Clin. Immunol.* 19: 1–11.
344. Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, and K. Hattori. 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378: 88–91.
345. Allan, S. M., P. J. Tyrrell, and N. J. Rothwell. 2005. Interleukin-1 and neuronal injury. *Nat. Rev. Immunol.* 5: 629–640.
346. Mertens, M., and J. A. Singh. 2009. Anakinra for rheumatoid arthritis: a systematic review. *J. Rheumatol.* 36: 1118–1125.
347. Dinarello, C. A. 2005. Blocking IL-1 in systemic inflammation. *Journal of Experimental Medicine* 201: 1355–1359.
348. Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proceedings of the National Academy of Sciences* 89: 12013–12017.
349. Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski. 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules. *J Immunol* 162: 5398–5406.
350. Harty, J. T., A. R. Tvinnereim, and D. W. White. 2000. CD8+ T cell effector mechanisms in resistance to infection. *Annu. Rev. Immunol.* 18: 275–308.
351. Chattopadhyay, A., N. Robinson, J. K. Sandhu, B. B. Finlay, S. Sad, and L. Krishnan. 2010. Salmonella enterica serovar Typhimurium-induced placental inflammation and not bacterial burden correlates with pathology and fatal maternal disease. *Infect. Immun.* 78: 2292–2301.
352. Kolumam, G. A., S. Thomas, L. J. Thompson, J. Sprent, and K. Murali-

- Krishna. 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *Journal of Experimental Medicine* 202: 637–650.
353. Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267: 2000–2003.
354. Dudani, R., Y. Chapdelaine, H. van Faassen, D. K. Smith, H. Shen, L. Krishnan, and S. Sad. 2002. Preexisting inflammation due to Mycobacterium bovis BCG infection differentially modulates T-cell priming against a replicating or nonreplicating immunogen. *Infect. Immun.* 70: 1957–1964.
355. Clarke, S. R., M. Barnden, C. Kurts, F. R. Carbone, J. F. Miller, and W. R. Heath. 2000. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunol. Cell Biol.* 78: 110–117.
356. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76: 17–27.
357. Xu, Y., Y. Zhan, A. M. Lew, S. H. Naik, and M. H. Kershaw. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J. Immunol.* 179: 7577–7584.
358. Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annu. Rev. Immunol.* 31: 563–604.
359. Helft, J., J. Böttcher, P. Chakravarty, S. Zelenay, J. Huotari, B. U. Schraml, D. Goubau, and C. Reis e Sousa. 2015. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity* 42: 1197–1211.
360. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *Journal of Experimental Medicine* 176: 1693–1702.
361. van de Laar, L., and B. N. Lambrecht. 2014. How to generate large numbers of CD103+ dendritic cells. *Blood* 124: 3036–3038.
362. Brasel, K., T. De Smedt, J. L. Smith, and C. R. Maliszewski. 2000. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 96: 3029–3039.
363. Mayer, C. T., P. Ghorbani, A. Nandan, M. Dudek, C. Arnold-Schrauf, C. Hesse, L. Berod, P. Stüve, F. Puttur, M. Merad, and T. Sparwasser. 2014. Selective and efficient generation of functional Batf3-dependent CD103+ dendritic cells from mouse bone marrow. *Blood* 124: 3081–3091.
364. Kaufmann, S. H. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* 11: 129–163.
365. Nauciel, C. 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection. *J. Immunol.* 145: 1265–1269.

366. Hess, J., C. Ladel, D. Miko, and S. H. Kaufmann. 1996. Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J Immunol* 156: 3321–3326.
367. Zijlstra, M., M. Bix, N. E. Simister, J. M. Loring, D. H. Raulet, and R. Jaenisch. 1990. Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature* 344: 742–746.
368. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051–1066.
369. Lee, S.-J., S. Dunmire, and S. J. McSorley. 2012. MHC class-I-restricted CD8 T cells play a protective role during primary Salmonella infection. *Immunol. Lett.* 148: 138–143.
370. Monack, D. M., D. M. Bouley, and S. Falkow. 2004. Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFN-gamma neutralization. *Journal of Experimental Medicine* 199: 231–241.
371. Bevan, M. J. 1995. Antigen presentation to cytotoxic T lymphocytes in vivo. *Journal of Experimental Medicine* 182: 639–641.
372. Ackerman, A. L., and P. Cresswell. 2004. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat. Immunol.* 5: 678–684.
373. Kerksiek, K. M., and E. G. Pamer. 1999. T cell responses to bacterial infection. *Curr. Opin. Immunol.* 11: 400–405.
374. Cossart, P., and P. J. Sansonetti. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* 304: 242–248.
375. Pancholi, P., A. Mirza, N. Bhardwaj, and R. M. Steinman. 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. *Science* 260: 984–986.
376. Gedde, M. M., D. E. Higgins, L. G. Tilney, and D. A. Portnoy. 2000. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect. Immun.* 68: 999–1003.
377. Dejager, L., S. Vandevyver, M. Ballegeer, E. Van Wonterghem, L.-L. An, J. Riggs, R. Kolbeck, and C. Libert. 2014. Pharmacological inhibition of type I interferon signaling protects mice against lethal sepsis. *J. Infect. Dis.* 209: 960–970.
378. Neefjes, J., and C. Sadaka. 2012. Into the intracellular logistics of cross-presentation. *Front Immunol* 3: 31.
379. Murphy, T. L., R. Tussiwand, and K. M. Murphy. 2013. Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks. *Nat. Rev. Immunol.* 13: 499–509.
380. Carrero, J. A., H. Vivanco-Cid, and E. R. Unanue. 2012. Listeriolysin o is strongly immunogenic independently of its cytotoxic activity. *PLoS ONE* 7: e32310.
381. Tussiwand, R., W.-L. Lee, T. L. Murphy, M. Mashayekhi, K. C. Wumesh, J. C. Albring, A. T. Satpathy, J. A. Rotondo, B. T. Edelson, N. M. Kretzer, X. Wu, L. A. Weiss, E. Glasmacher, P. Li, W. Liao, M. Behnke, S. S. K. Lam, C. T. Aurthur, W. J. Leonard, H. Singh, C. L. Stallings, L. D. Sibley, R. D. Schreiber, and K. M.

- Murphy. 2012. Compensatory dendritic cell development mediated by BATF-IRF interactions. *Nature* 490: 502–507.
382. Winau, F., S. Weber, S. Sad, J. de Diego, S. L. Hoops, B. Breiden, K. Sandhoff, V. Brinkmann, S. H. E. Kaufmann, and U. E. Schaible. 2006. Apoptotic Vesicles Crossprime CD8 T Cells and Protect against Tuberculosis. *Immunity* 24: 105–117.
383. van der Velden, A. W. M., M. Velasquez, and M. N. Starnbach. 2003. Salmonella rapidly kill dendritic cells via a caspase-1-dependent mechanism. *J. Immunol.* 171: 6742–6749.
384. Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* 22: 240–73– Table of Contents.
385. Rathinam, V. A. K., S. K. Vanaja, and K. A. Fitzgerald. 2012. Regulation of inflammasome signaling. *Nat. Immunol.* 13: 333–332.
386. Wu, Y.-T., H.-L. Tan, Q. Huang, X.-J. Sun, X. Zhu, and H.-M. Shen. 2011. zVAD-induced necroptosis in L929 cells depends on autocrine production of TNF α mediated by the PKC-MAPKs-AP-1 pathway. *Cell Death Differ.* 18: 26–37.
387. Li, J.-X., J.-M. Feng, Y. Wang, X.-H. Li, X.-X. Chen, Y. Su, Y.-Y. Shen, Y. Chen, B. Xiong, C.-H. Yang, J. Ding, and Z.-H. Miao. 2014. The B-RafV600E inhibitor dabrafenib selectively inhibits RIP3 and alleviates acetaminophen-induced liver injury. *Cell Death and Disease* 5: e1278–11.
388. Labbé, K., C. R. McIntire, K. Doiron, P. M. Leblanc, and M. Saleh. 2011. Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. *Immunity* 35: 897–907.
389. Vince, J. E., W. W.-L. Wong, I. Gentle, K. E. Lawlor, R. Allam, L. O'Reilly, K. Mason, O. Groß, S. Ma, G. Guarda, H. Anderton, R. Castillo, G. Häcker, J. Silke, and J. Tschopp. 2012. Inhibitor of Apoptosis Proteins Limit RIP3 Kinase-Dependent Interleukin-1 Activation. *Immunity* 36: 215–227.
390. Hume, D. A. 2008. Macrophages as APC and the dendritic cell myth. *The Journal of Immunology* 181: 5829–5835.
391. Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto. 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol.* 1: 311–316.
392. Heath, W. R., and F. R. Carbone. 2009. Dendritic cell subsets in primary and secondary T cell responses at body surfaces. *Nat. Immunol.* 10: 1237–1244.
393. Mildner, A., and S. Jung. 2014. Development and function of dendritic cell subsets. *Immunity* 40: 642–656.
394. Steinman, R. M., and Z. A. Cohn. 2007. *Pillars Article: Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution.* *J. Exp. Med.* 1973. 137: 1142-1162. :5–25.
395. Steinman, R. M., and Z. A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *Journal of Experimental Medicine* 139: 380–397.
396. Dinarello, C. A. 2009. Immunological and Inflammatory Functions of the Interleukin-1 Family. *Annu. Rev. Immunol.* 27: 519–550.
397. Latz, E., T. S. Xiao, and A. Stutz. 2013. Activation and regulation of the inflammasomes. *Nat. Rev. Immunol.* 13: 397–411.

398. Ashida, H., H. Mimuro, M. Ogawa, T. Kobayashi, T. Sanada, M. Kim, and C. Sasakawa. 2011. Cell death and infection: a double-edged sword for host and pathogen survival. *J. Cell Biol.* 195: 931–942.
399. Khan, R. T., K. E. Yuki, and D. Malo. 2014. Fine-mapping and phenotypic analysis of the *Ity3* Salmonella susceptibility locus identify a complex genetic structure. *PLoS ONE* 9: e88009.
400. Griffin, A. J., and S. J. McSorley. 2011. Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* 4: 371–382.
401. Ravindran, R., and S. J. McSorley. 2005. Tracking the dynamics of T-cell activation in response to Salmonella infection. *Immunology* 114: 450–458.
402. McSorley, S. J., B. T. Cookson, and M. K. Jenkins. 2000. Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium. *J. Immunol.* 164: 986–993.
403. Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- Salmonella vaccines. *Microb. Pathog.* 13: 477–491.
404. Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. *Infect. Immun.* 61: 3981–3984.
405. Collins, H. L., and S. H. Kaufmann. 2001. The many faces of host responses to tuberculosis. *Immunology* 103: 1–9.
406. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat. Rev. Immunol.* 6: 476–483.
407. Alaniz, R. C., S. Sandall, E. K. Thomas, and C. B. Wilson. 2004. Increased dendritic cell numbers impair protective immunity to intracellular bacteria despite augmenting antigen-specific CD8+ T lymphocyte responses. *J Immunol* 172: 3725–3735.
408. Hervas-Stubbs, S., J. L. Perez-Gracia, A. Rouzaut, M. F. Sanmamed, A. Le Bon, and I. Melero. 2011. Direct Effects of Type I Interferons on Cells of the Immune System. *Clin. Cancer Res.* 17: 2619–2627.
409. Diamond, M. S., M. Kinder, H. Matsushita, M. Mashayekhi, G. P. Dunn, J. M. Archambault, H. Lee, C. D. Arthur, J. M. White, U. Kalinke, K. M. Murphy, and R. D. Schreiber. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J. Exp. Med.* 208: 1989–2003.
410. Schiavoni, G., F. Mattei, and L. Gabriele. 2013. Type I Interferons as Stimulators of DC-Mediated Cross-Priming: Impact on Anti-Tumor Response. *Front Immunol* 4: 483.
411. del Rio, M.-L., J.-I. Rodriguez-Barbosa, E. Kremmer, and R. Förster. 2007. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J. Immunol.* 178: 6861–6866.
412. Ashok, D., S. Schuster, C. Ronet, M. Rosa, V. Mack, C. Lavanchy, S. F. Marraco, N. Fasel, K. M. Murphy, F. Tacchini-Cottier, and H. Acha-Orbea. 2014.

- Cross-presenting dendritic cells are required for control of *Leishmania major* infection. *Eur. J. Immunol.* 44: 1422–1432.
413. Martínez-López, M., S. Iborra, R. Conde-Garrosa, and D. Sancho. 2015. Batf3-dependent CD103⁺ dendritic cells are major producers of IL-12 that drive local Th1 immunity against *Leishmania major* infection in mice. *Eur. J. Immunol.* 45: 119–129.
414. McComb, S., E. Cessford, N. A. Alturki, J. Joseph, B. Shutinoski, J. B. Startek, A. M. Gamero, K. L. Mossman, and S. Sad. 2014. Type-I interferon signaling through ISGF3 complex is required for sustained Rip3 activation and necroptosis in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 111: E3206–13.
415. Chawla-Sarkar, M., D. J. Lindner, Y.-F. Liu, B. R. Williams, G. C. Sen, R. H. Silverman, and E. C. Borden. 2003. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 8: 237–249.
416. Allan, R. S., J. Waithman, S. Bedoui, C. M. Jones, J. A. Villadangos, Y. Zhan, A. M. Lew, K. Shortman, W. R. Heath, and F. R. Carbone. 2006. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 25: 153–162.
417. Carbone, F. R., G. T. Belz, and W. R. Heath. 2004. Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. *Trends Immunol.* 25: 655–658.
418. Yatim, N., and M. L. Albert. 2011. Dying to replicate: the orchestration of the viral life cycle, cell death pathways, and immunity. *Immunity* 35: 478–490.
419. Dolan, B. P., K. D. Gibbs, and S. Ostrand-Rosenberg. 2006. Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8⁺ T cells. *J. Immunol.* 177: 6018–6024.
420. Li, L., S. Kim, J. M. Herndon, P. Goedegebuure, B. A. Belt, A. T. Satpathy, T. P. Fleming, T. H. Hansen, K. M. Murphy, and W. E. Gillanders. 2012. Cross-dressed CD8 α ⁺/CD103⁺ dendritic cells prime CD8⁺ T cells following vaccination. *Proc Natl Acad Sci USA* 109: 12716–12721.
421. McLellan, A. D., M. Kapp, A. Eggert, C. Linden, U. Bommhardt, E.-B. Bröcker, U. Kämmerer, and E. Kämpgen. 2002. Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood* 99: 2084–2093.
422. Tailor, P., T. Tamura, H. C. Morse, and K. Ozato. 2008. The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. *Blood* 111: 1942–1945.
423. Grajales-Reyes, G. E., A. Iwata, J. Albring, X. Wu, R. Tussiwand, W. KC, N. M. Kretzer, C. G. Briseño, V. Durai, P. Bagadia, M. Haldar, J. Schönheit, F. Rosenbauer, T. L. Murphy, and K. M. Murphy. 2015. Batf3 maintains autoactivation of Irf8 for commitment of a CD8 α ⁺ conventional DC clonogenic progenitor. *Nat. Immunol.* 16: 708–717.
424. Sundquist, M., and M. J. Wick. 2009. Salmonella induces death of CD8 α ⁺ dendritic cells but not CD11c(int)CD11b⁺ inflammatory cells in vivo via MyD88 and TNFR1. *J Leukoc Biol* 85: 225–234.
425. Niedergang, F., J. C. Sirard, C. T. Blanc, and J. P. Kraehenbuhl. 2000. Entry and survival of *Salmonella typhimurium* in dendritic cells and presentation of

recombinant antigens do not require macrophage-specific virulence factors. *Proc Natl Acad Sci USA* 97: 14650–14655.

426. Schnorrer, P., G. M. N. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, G. T. Belz, F. R. Carbone, K. Shortman, W. R. Heath, and J. A. Villadangos. 2006. The dominant role of CD8⁺ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci USA* 103: 10729–10734.

427. Zhang, Y., H. Yoneyama, Y. Wang, S. Ishikawa, S.-I. Hashimoto, J.-L. Gao, P. Murphy, and K. Matsushima. 2004. Mobilization of dendritic cell precursors into the circulation by administration of MIP-1alpha in mice. *J. Natl. Cancer Inst.* 96: 201–209.

428. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29: 1617–1625.

429. Sundquist, M., and M. J. Wick. 2005. TNF-alpha-dependent and -independent maturation of dendritic cells and recruited CD11c(int)CD11b⁺ Cells during oral Salmonella infection. *J. Immunol.* 175: 3287–3298.

430. Coburn, B., I. Sekirov, and B. B. Finlay. 2007. Type III secretion systems and disease. *Clin. Microbiol. Rev.* 20: 535–549.

431. Blachère, N. E., R. B. Darnell, and M. L. Albert. 2005. Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. *PLoS Biol.* 3: e185.

432. Ravichandran, K. S. 2010. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J. Exp. Med.* 207: 1807–1817.

433. Elliott, M. R., F. B. Chekeni, P. C. Trampont, E. R. Lazarowski, A. Kadl, S. F. Walk, D. Park, R. I. Woodson, M. Ostankovich, P. Sharma, J. J. Lysiak, T. K. Harden, N. Leitinger, and K. S. Ravichandran. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 461: 282–286.

434. Truman, L. A., C. A. Ford, M. Pasikowska, J. D. Pound, S. J. Wilkinson, I. E. Dumitriu, L. Melville, L. A. Melrose, C. A. Ogden, R. Nibbs, G. Graham, C. Combadiere, and C. D. Gregory. 2008. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* 112: 5026–5036.

435. Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148: 2207–2216.

436. Lauber, K., S. G. Blumenthal, M. Waibel, and S. Wesselborg. 2004. Clearance of apoptotic cells: getting rid of the corpses. *Molecular Cell* 14: 277–287.

437. Fink, S. L., and B. T. Cookson. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect. Immun.* 73: 1907–1916.

438. Yatim, N., H. Jusforgues-Saklani, S. Orozco, O. Schulz, R. Barreira da Silva, C. Reis e Sousa, D. R. Green, A. Oberst, and M. L. Albert. 2015. RIPK1

- and NF- κ B signaling in dying cells determines cross-priming of CD8⁺ T cells. *Science* 350: 328–334.
439. Tsuji, N. M., H. Tsutsui, E. Seki, K. Kuida, H. Okamura, K. Nakanishi, and R. A. Flavell. 2004. Roles of caspase-1 in *Listeria* infection in mice. *Int. Immunol.* 16: 335–343.
440. Lara-Tejero, M. 2006. Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J. Exp. Med.* 203: 1407–1412.
441. Berghe, T. V., A. Linkermann, S. Jouan-Lanhouet, H. Walczak, and P. Vandenabeele. 2014. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat. Rev. Mol. Cell Biol.* 15: 135–147.
442. Lamkanfi, M. 2011. Emerging inflammasome effector mechanisms. *Nat. Rev. Immunol.* 11: 213–220.
443. Jorgensen, I., and E. A. Miao. 2015. Pyroptotic cell death defends against intracellular pathogens. *Immunol. Rev.* 265: 130–142.
444. Newton, K. 2015. RIPK1 and RIPK3: critical regulators of inflammation and cell death. *Trends in Cell Biology* 25: 347–353.
445. Kayagaki, N., S. Warming, M. Lamkanfi, L. V. Walle, S. Louie, J. Dong, K. Newton, Y. Qu, J. Liu, S. Heldens, J. Zhang, W. P. Lee, M. Roose-Girma, and V. M. Dixit. 2012. Non-canonical inflammasome activation targets caspase-11. *Nature* 479: 117–121.
446. Viganò, E., and A. Mortellaro. 2013. Caspase-11: The driving factor for noncanonical inflammasomes. *Eur. J. Immunol.* 43: 2240–2245.
447. Broz, P., T. Ruby, K. Belhocine, D. M. Bouley, N. Kayagaki, V. M. Dixit, and D. M. Monack. 2012. Caspase-11 increases susceptibility to *Salmonella* infection in the absence of caspase-1. *Nature* 490: 288–291.
448. Meunier, E., M. S. Dick, R. F. Dreier, N. Schürmann, D. Kenzelmann Broz, S. Warming, M. Roose-Girma, D. Bumann, N. Kayagaki, K. Takeda, M. Yamamoto, and P. Broz. 2014. Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* 509: 366–370.
449. Meunier, E., P. Wallet, R. F. Dreier, S. Costanzo, L. Anton, S. Rühl, S. Dussurgey, M. S. Dick, A. Kistner, M. Rigard, D. Degrandi, K. Pfeffer, M. Yamamoto, T. Henry, and P. Broz. 2015. Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella novicida*. *Nature Publishing Group* 16: 476–484.
450. Green, D. R. 2011. Immunology: A heavyweight knocked out. *Nature* 479: 48–50.
451. Kang, T.-B., S.-H. Yang, B. Toth, A. Kovalenko, and D. Wallach. 2013. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* 38: 27–40.
452. Philip, N. H., C. P. Dillon, A. G. Snyder, P. Fitzgerald, M. A. Wynosky-Dolfi, E. E. Zwack, B. Hu, L. Fitzgerald, E. A. Mauldin, A. M. Copenhaver, S. Shin, L. Wei, M. Parker, J. Zhang, A. Oberst, D. R. Green, and I. E. Brodsky. 2014. Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF- κ B and MAPK signaling. *Proceedings of the National Academy of Sciences* 111: 7385–7390.
453. Man, S. M., P. Tourlomousis, L. Hopkins, T. P. Monie, K. A. Fitzgerald, and

C. E. Bryant. 2013. Salmonella Infection Induces Recruitment of Caspase-8 to the Inflammasome To Modulate IL-1 β Production. *J. Immunol.* 191: 5239–5246.