Immunological checkpoints in the control of murine *Salmonella enterica* infection: IFN-γ pathways and early dendritic cell death

Chenying Yang

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Department of Microbiology and Immunology
The University of Melbourne

Abstract

Salmonella enterica is a Gram-negative intracellular pathogen, which can cause typhoid fever and non-typhoidal salmonellosis. Every year ~22 million cases and ~200,000 deaths are reported for typhoid fever and ~93 million cases and ~155,000 deaths for non-typhoidal salmonellosis. Innate immunity provides the very early protection against *Salmonella,* a better understanding of which may lead to a progress in treatment and prevention to *Salmonella* infection. Dendritic cells (DC) are one of the first cells to sense *Salmonella in vivo*, and play an important role in initiating a cascade of innate immune control, including phagocytising bacteria and the activation of inflammasomes, which further induces cell death and the production of pro-inflammatory cytokines, such as IFN-γ. In addition, dendritic cells are potent antigen presenting cells (APC) that induce the development of protective adaptive immunity against *Salmonella*. However, it has been reported that *Salmonella* possesses various mechanisms, including regulating phagolysosomal fusion and delaying vacuole acidification, and down-regulating flagellin expression to prevent antigen presentation, highlighting the dynamic and complex nature of DC-*Salmonella* interactions. In recent years, the critical role of DCs in immunity against *Salmonella* has gained increased attention, however the cellular and molecular mechanisms of the DC-*Salmonella* interactions are not fully understood.

The first aim of this study was to study the survival and death in infected DCs during *Salmonella* infection, utilising murine bone marrow-derived DCs (BMDCs), which are sensitive to *Salmonella*-induced cell death within hours of infection. It is found that several virulence factors such as lipopolysaccharide (LPS), Type III secretion system 1 (SPI-1) and flagellin contribute to, and in combination maximise, death in BMDCs. Intriguingly, BMDCs that were not directly infected with *Salmonella* were killed upon infection of neighbouring cells in culture. An apparently similar 'bystander' cell death was induced by co-culturing with filtered supernatant from infected BMDCs, suggesting a role for contact-independent mechanisms. Infected BMDCs released several cytokines, including IL-6, MCP-1 and TNF-α. However, blockade of intracellular protein transport and secretion of cytokines by monesin did not alter *Salmonella*-induced cell death in uninfected bystanders, suggesting that the

bystander effect is not dependent on mediators released from infected BMDCs. BMDCs from mice with gene knockouts in key pathways that are involved in DC immune responses against *Salmonella* were also tested, and decreased death was observed in $ICE^{-/-}$ BMDCs, suggesting that caspase-1/caspase-11-mediated pyroptosis could be responsible for direct as well as bystander BMDC death.

The second aim was to determine the contribution of IFN-γ and the IFN-γ induction pathways in *Salmonella* infection. Previous studies in our lab have shown that flagellin-induced NLRC4 inflammasome activation in splenic DCs triggers non-cognate memory $CD8^+$ T cells to produce IFN-γ, a critical mediator of innate immunity against *Salmonella*. It was shown in the present study that deletion of individual components of the NLRC4 inflammasome pathway, e.g. caspase-1 or IL-18, can lead to a moderate reduction of IFN-γ production, but the impact on the control of *Salmonella* in infected mice is minimal, suggesting that NLRC4 pathway is not the only source of IFN-γ and that low level of IFN-γ may be sufficient for full protection against *Salmonella*. In the studies presented here, it was shown that LPS-induced activation of the TLR4 pathway is also an important source of IFN-γ and that mice deficient in components of TLR4 pathway has poor early control of bacterial load during *S.* Typhimurium BRD509 infection. Interestingly, deficiency in the TLR4 pathway led to an increase rather than reduction of IFN-γ, suggesting that IFN-γ is regulated by different pathways and that TLR4 pathway may be involved in other immune responses that are important for early control of *Salmonella*.

Declaration

This is to certify that

- the thesis comprises only my original work towards the MPhil except where indicated in the text,

- due acknowledgement has been made in the text to all other material used, and

- the thesis is less than 50,000 words in length, exclusive of tables, figures, bibliographies and appendices.

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Chenying Yang

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Abbreviations

°C	Degrees Celsius
%	Percent
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
	Allophycocyanin
APC-Cy7	Allophycocyanin cyanine 7
ASC	Apoptosis-associated speck-like protein containing CARD
B6	C57BL/6
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
BRF	Biological Research Facility
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Cluster of differentiation
CO_2	Carbon dioxide
CTV	CellTrace Violet
cDC	Conventional dendritic cell
cfu	Colony forming unit
DC	Dendritic cell
DNA	Deoxyribonucleic acid d
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular Signal-regulated Kinase
e.g.	*exempli gratia* (for example)
et al.	*et alii* (and others)
etc.	*et cetera* (and the rest)
FCS	Fetal calf serum
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocynate
FLT-3	Fms-like tyrosine kinase 3
G	Gauge
× g	Gravitational acceleration on earth (unit for centrifugation speed)
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hours
HIV	Human Immunodeficiency Virus

HKST	Heat-killed *Salmonella* Typhimurium
ICE	Interleukin-1β-converting enzyme
IFNAR	interferon-α/β receptor
IFN-γ	Interferon gamma
IFN-gR	Interferon gamma receptor
IL	Interleukin
IRAK-4	Interleukin-1 receptor-associated kinase 4
iNTS	Invasive non-typhoidal Salmonellosis
iNOS	Inducible nitric oxide synthase
i.e.	*id est* (that is)
i.p.	Intraperitoneal
i.v.	Intravenous
LB	Luria Broth
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
M cell	Microfold cell
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte chemotractant protein 1
MLN	Mesenteric lymph nodes
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MOI	Multiplicities of infection
MyD88	Myeloid differentiation primary response gene 88
min	Minute
mM	Millimolar
moDC	monocyte-derived dendritic cell
NLR	Nod-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NLR family pyrin domain containing protein 3
NK cell	Natural killer cell
NO	Nitric oxide
Nramp1	Natural resistance-associated macrophage protein 1
NTS	Non-typhoidal Salmonellosis
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin

PFA	paraformaldehyde
PI	Propidium iodide
PP	Peyers patch
PRR	Pattern recognition receptor
pDC	Plasmacytoid dendritic cell
Rag	Recombinase activating gene
RBC	Red blood cell
RIP	Receptor-interacting protein
RIPK	Receptor-interacting protein kinase
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RT	Room temperature
SCV	*Salmonella*-containing vacuoles
SPF	Specific pathogen free
SPI	*Salmonella* pathogenicity island
spp.	Plural species
STAT	Signal transducers and activators of transcription
STM	*Salmonella* Typhimurium
T3SS	Type three secretion system
TAB	TAK1-binding protein
TAC	Tris-ammonium chloride
TAK1	TGF-β-activated kinase 1
TCR	T cell receptor
Th	T helper
TLR	Toll like receptor
TNF-α	Tumour necrosis factor α
TRAF	TNF receptor associated factor
TRIF	Tir-domain-containing adaptor-inducing interferon beta
U	Units
µM	Micromolar
µm	Micrometre
WHO	World Health Organization
WT	Wild type

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Chapter 1

Introduction and Literature Review

1.1 *Salmonella* spp.

The *Salmonella* species are Gram-negative facultative intracellular bacteria that cause diseases such as enterocolitis and typhoid fever in humans, and a range of conditions in other animals[1-3]. The genus *Salmonella*, a member of the family of *Enterobacteriaceae,* can be divided into two species, *Salmonella enterica* and *Salmonella bongori. Salmonella enterica* consists of 6 subspecies, which are further classified into different serovars by the surface antigens O (somatic/lipopolysaccharide (LPS)), H (flagella) and K (capsular)[4]. Based on the Kauffmann-White scheme, the nomenclature of *Salmonella* is "Genus-Species-Subspecies-Serovar", but commonly shortened as "Genus-Serovar"[5].

Salmonella enterica is the major cause of salmonellosis in humans worldwide[6]. The clinical manifestation caused by *Salmonella* infection varies depending on the serovar. Typhoid fever is generally regarded as the most severe *Salmonella* infection and is caused by serovar Typhi. A typhoid fever-like disease is caused by serovars Paratyphi A, B and C. Milder, but increasingly more prevalent disease, gastroenteritis, is predominantly caused by serovar Typhimurium. In recent years, highly virulent, and often lethal *Salmonella* infections can be caused by gastroenteritis serovars such as Typhimurium. This emerging disease, termed invasive Non-Typhoidal Salmonellosis (iNTS), has been observed in sub-Saharan Africa, and is often in association with Human Immunodeficiency Virus (HIV) infection[7-12].

1.2 Host Specificity of *Salmonella*

Salmonella enterica serovars can be classified into three groups according to the host range: host-specific, host-restricted and ubiquitous. The first group contains serovars that cause systemic diseases only in a few closely related species. For instance, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) causes disease in humans and some other primates[13]. There are other *Salmonella* serovars that are also restricted to one or a small number of host species[2]. These strains include *Salmonella enterica* serovar Dublin in cattle and *Salmonella enterica* serovar Gallinarium in poultry[14, 15]. The second group of serovars contains bacteria that cause diseases mainly in several closely related species but occasionally in other animals[16]. The third group includes strains that usually lead to gastroenteritis in a broad range of species.

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is an example. It may be a commensal in poultry[17, 18], case gastroenteritis in humans[19, 20], a typhoid-like systemic disease in mice[21, 22] and other syndromes in cattle[23-25]. The influence of *Salmonella* infections extends beyond human health—infection in animals, especially food animals, can have an economic impact[26, 27].

1.3 Clinical Relevance of *Salmonella*

1.3.1 Typhoid Fever

Typhoid fever was a big problem in the 19th century due to poor sanitation and hygiene conditions. With the improvement in sanitation and the introduction of antibiotic treatment, the incidence of typhoid fever in developed countries dramatically decreased. However, typhoid fever is still endemic in Africa and Asia. According to the estimation of World Health Organization (WHO) in 1997, 16 million cases and 600,000 deaths occur every year[28] and a more recent estimation claimed that typhoid fever caused 27 million cases and 220,000 deaths annually[29]. Deficiencies in sanitation facilities in these developing countries, and difficulty in diagnosis due to lack of typical clinical signs, make accurate data of typhoid fever hard to acquire[30-32], which suggests that the incidence of typhoid fever is still grossly underestimated. Additionally, the usage of antibiotics has applied selection pressure to *S.* Typhi and resulted in antibiotic resistance, which potentially leads to increasing level of difficulty in treatment and can drive wider spreading of infection[33].

1.3.2 Pathogenesis of typhoid fever

Foodborne *Salmonella* is typically ingested with food or water. After surviving gastric acid, the bacteria reach the intestine and adhere to mucosal cells[34]. *S.* Typhi then interacts and penetrates the mucosal epithelia, especially microfold cells (M cells), which are specialised epithelial cells in the epithelial barrier that present antigens in the gut to immune cells[35]. *In vitro* studies have shown that this invasion is mediated by *Salmonella* pathogenicity island (SPI)-1, which encodes a type III secretion system (T3SS); a needle-like complex that delivers bacterial proteins into the host cell cytosol[36]. Once through this barrier, *Salmonella* invade lymphoid tissues such as the

intestinal lymphoid follicles and draining mesenteric lymph nodes[34], where they are taken up by immune cells[37]. As *Salmonella* have acquired the ability to invade and survive within immune cells, they can replicate in the organs that are rich in immune cells, such as lymph nodes, spleen and liver. The invasion and survival mechanisms include: regulating phagolysosomal fusion and delaying vacuole acidification[38, 39], and down-regulating flagellin expression to prevent this antigen from being recognised by dendritic cells (DCs)[40]. After an incubation period of 7 to 14 days, a secondary bacteraemia that may last for several weeks is observed. During this stage, bacteria disseminate widely through lymphatic and blood vessels to the liver, spleen, bone marrow and gall bladder[41]. Patients will usually display a fever that progressively rises within the first week[42]. Other symptoms including malaise, anorexia, abdominal discomfort and myalgia may appear. The physical signs include hepatomegaly, splenomegaly, tender abdomen and coated tongue[43]. If treated with appropriate antibiotics, the fever reduces slowly within 3-4 days, otherwise, it may persist for another several weeks and such patients typically suffer weight loss, weakness, gastrointestinal bleeding, intestinal perforation, typhoid encephalopathy and a poor mental state[42, 44].

1.3.3 Non-typhoidal Salmonellosis (NTS) and iNTS infection

Non-typhoidal serovars of *Salmonella* are known to be a major cause of foodborne gastroenteritis. It is estimated that NTS are responsible for 93.8 million cases with 155,000 deaths annually across the world, of which, 80.3 million cases were estimated to be foodborne[20]. The presence of NTS in food animals such as poultry, cattle and pigs results in contamination in eggs, milk and meat, which serve as the major vehicles of infection[45-48]. The symptoms of non-typhoidal salmonellosis usually include: diarrhoea, abdominal pain, nausea and fever, depending on the syndrome, i.e. gastroenteritis or sepsis. For most healthy individuals, the bacteria are usually gut-localised and invasion beyond the gastrointestinal tract is rare[49].

However, NTS, most commonly *S. enterica* serovars Typhimurium and Enteritidis, may also cause iNTS infection, a systemic bacteraemia without diarrhoea, in malaria patients and immunocompromised people, such as diabetic patients and those with acquired immunodeficiency syndrome (AIDS)[8, 20, 50]. iNTS infections are most

commonly reported in Africa, where malaria, malnutrition and HIV are more prevalent[8]. The annual global burden of iNTS is estimated to be 2000–7500 cases per 100,000 HIV-infected adults and 175–388 cases per 100,000 children aged 3–5 years with poor nutrition[51-53], with a fatality rate of 20-25%[53].

The currently licensed vaccines for salmonellosis demonstrate limited efficacy and duration of protection, and are restricted, in humans, to the prevention of typhoid fever only[54-61]. A thorough understanding of immunity to *Salmonella* infection for generating alternative treatments is both necessary and urgent.

1.4 Mouse models for typhoid fever

To study typhoid fever in mice, *S.* Typhimurium is used and the resulting disease in mice resembles typhoid fever in humans in that it has a bacteraemia component. This mouse model has been used since the 19th century and is used in the present study to further understand innate immunity during salmonellosis.

Nramp-1 (natural resistance-associated macrophage protein 1) or Slc11a1 (solute carrier family 11 member 1), a protein transporter of iron and manganese out from phagolysosomes to make the environment of phagolysosomes less suited for bacteria growth, affects the host control of intracellular replication of *Salmonella* and determines the outcome of *S.* Typhimurium infection in mice[62, 63]. Mouse strains that are commonly used, such as C57BL/6 (B6) or BALB/c, possess a single point mutation resulting in an amino acid change in *Nramp1*, which results in the disability to express mature Nramp1 protein[64]. These mice are highly susceptible to wild type *S.* Typhimurium infection and succumb within 6-8 days[65, 66]. Infected B6 mice show signs of systemic infection including weight loss, hepatomegaly, splenomegaly and high bacterial load in the liver and spleen[67].

Salmonella strains deficient in some, distinct metabolic pathways have problems in acquiring sufficient nutrients[68] and, as such, grow much more slowly. This leads to a reduction in their virulence[69]. Attenuated strains, such as BRD509 (reported as Δ*aroA*Δ*aroD*), are used to model infection as it causes milder disease, which extends the infection period to allow the full course of anti-*Salmonella* immunity to be

studied[70, 71]. Infections of attenuated strains in WT mice follow a typical pattern (Fig.1-1): 1. a short decrease in bacterial count, which is most likely the result of reactive oxygen species (ROS)[72], 2. an exponential growth, which can be affected by innate immunity, 3. a plateau phase, in which cytokines, such as IFN-γ are likely to be involved[71] and 4. clearance of bacteria. In this clearance of *Salmonella*, adaptive immune system components are involved[71, 73]. It was shown that mice deficient in $CD4^+$ T cells (GK1.5Tg or $IAE^{-/-}$) were not able to clear bacteria and developed a chronic infection; mice deficient in $CD4^+$ and $CD8^+$ T cells $(GK1.5Tg \times 2.43Tg)_{F1}$ and mice deficient in B cells and T cells ($Rag1/Je^{-/-}$) succumbed to the *S.* Typhimurium infection at this stage[71].

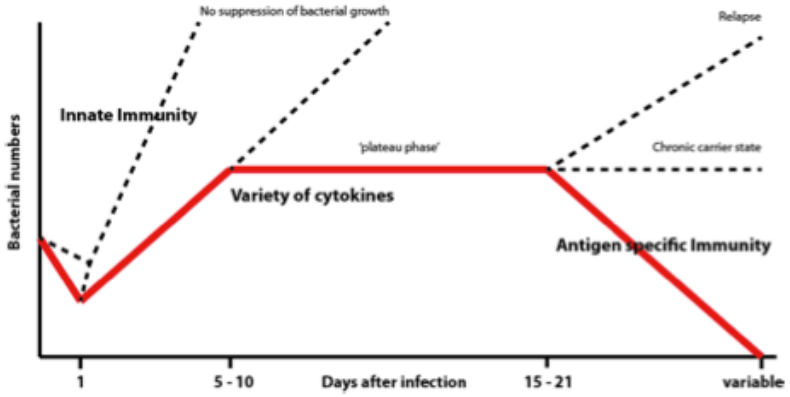

Figure 1-1: mouse model of attenuated Salmonella infection.

Bacterial growth in fully immunocompetent mice, such as B6, follow the pattern shown as the solid red line, while growth in immonocompromised mice may follow the dotted lines according to different mechanisms[41]. The figure is taken from P. Mastroeni 'Mechanisms of immunity to Salmonella infections' in Salmonella infections Clinical, Immunological and Molecular Aspects, Cambridge University Press (2006).

1.5 Innate immunity in *Salmonella* infection

The innate immune system, as the first line of host defence, involves many components and provides rapid but non-specific protection and non-enhancible protection (i.e. through vaccination) against pathogens[74]. The immune system senses and recognises pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), such as TLRs and Nod-like receptors (NLRs), and initiates a serial of immune reactions[75-77]. Cytoplasmic NLRs recognise PAMPs in the

cytosol of host cells, while membrane-bound TLRs respond to extracellular or endosomal determinants[78, 79]. In *Salmonella* infections, many mechanisms and immune cells participate in the process of host defense[80] and this study will focus on the role of dendritic cells (DCs) in *Salmonella* infection as DCs sit at the interface between innate and adaptive immunity[81-83].

1.6 DCs in *Salmonella* infection

Dendritic cells originate from hematopoietic bone marrow precursors and share a characteristic feature of producing dendrites[83]. DCs are present in both lymphoid and non-lymphoid tissues[84] and are one of the first cells to sense *Salmonella in vivo*, thus playing an important role in initiating a cascade of innate immune control[81, 82]. After recognising the pathogen through PRRs, DCs phagocyte and degrade the bacteria[85, 86]. Also, as antigen presenting cells (APC), DCs migrate to the lymph nodes and present pathogen-derived antigens to T cells via major histocompatibility complex (MHC) molecules[87-91]. The immature DCs, usually located in peripheral tissues, possess high capacity for phagocytosis and express low level of co-stimulate molecules, including CD80, CD86 and CD40, which are needed for activating naïve T cells[92-95]. Once DCs encounter the pathogen, the pathogen PAMPs are recognised by pattern recognition receptors of DCs, which then actively secrete anti-microbial molecules and pro-inflammatory cytokines, including IL-12 and IL-18, which activate IFN-γ production in NK cells and T cells[82]. In this process, DCs are activated and undergo maturation, after which the phagocytic capacity of DCs is reduced and the expressed levels of co-stimulate molecules is increased[90, 94-97]. Mature DCs then migrate from the infection site to the lymph nodes and spleen where naïve T cells locate[98].

Nevertheless, as outlined, *S.* Typhimurium has evolved an ability to interfere with, and prevent DCs from, functioning properly. Research has shown that *S.* Typhimurium down-regulate flagellin expression to avoid detection by DCs[40], presumably responsible for the limited activation of flagellin-specific T cells. B6 mice infected with a *S.* Typhimurium strain that constantly expresses flagellin survived after infection, while the mice infected by wild type (WT) *S.* Typhimurium died around 7 days post infection[99]. Aflagellated *S.* Typhimurium causes more severe systemic infection than WT strains[100, 101]. Following ingestion by DCs through

phagocytosis or active invasion, *S.* Typhimurium regulates phagolysosomal fusion and delays vacuole acidification to survive inside DCs[38, 39].

Intracellular *S.* Typhimurium reside within *Salmonella*-containing vacuoles (SCV), where the bacteria can survive and replicate without being detected by the host[102-104]. The formation of SCV varies slightly in different cell types, but always involves an acquisition of early endosome molecules and recycling of the endocytic markers[105, 106]. A protein of *S.* Typhimurium, SpiC was found to be vital in preventing SCV from fusing with lysosomes. SpiC binds to host protein Hook3, which is a bridge between the Golgi apparatus and the microtubules, to interfere with vesicular trafficking in DCs[102, 107, 108]. Another protein found to be crucial in the intracellular replication process is SifA, which participates in the formation of filaments induced by *Salmonella. S.* Typhimurium that lack SifA are unable to maintain the integrity of SCV[109-111].

Research also showed that *Salmonella* is capable of manipulating the MHC molecules expressed by DCs via SPI-II encoded T3SS[112, 113]. As the consequence of these mechanisms of *Salmonella*, the host has limited access to bacterial proteins and the antigen presenting function of DCs is subverted.

1.7 Programmed cell death caused by *Salmonella* infection

1.7.1 Apoptosis in *Salmonella* infection

In addition to subverting function of DCs, *Salmonella* is also able to cause programmed cell death in host cells including DCs via variable mechanisms[114, 115]. *In vitro* studies have shown that *Salmonella* can induce cell death in epithelial cells[116]; in the death of epithelial cells, caspase-3 and caspase-8 activation, DNA cleavage and nuclear condensation were demonstrated[116-118]. This suggests that *S.* Typhimurium is capable of inducing apoptosis in the epithelia. Apoptosis can be induced by signals transmitted extracellularly and intracellularly[119]. The extrinsic pathway is induced through activation of death receptors on the cell surface, such as the Tumour necrosis factor (TNF) receptor, which leads to caspase-8 activation[120]. The intrinsic pathway is

initiated by the release of cytochrome c, which activates caspase-9[121]. Caspase-8 and caspase-9 both can activate effector caspase-3 and eventually lead to apoptosis[122-124]. Features of apoptosis include: cell shrinkage, condensed cytoplasm and chromatin, DNA degradation, blebbing of the cell membrane and formation of apoptotic bodies[125].

1.7.2 Pyroptosis in *Salmonella* infection

Salmonella also induces a different form of programmed cell death called pyroptosis, which is mediated via caspase-1 activation, driven by the detection of flagellin by the NLRC4 inflammasome[126-128]. Control of bacterial replication can be mediated via caspase-1 induced macrophage death independent of IL-1b or IL-18[99]. In *Salmonella*-infected macrophages, cell lysis, release of pro-inflammatory cytokines and mature forms of IL-1b and IL-18 are observed[129]. The effector of apoptosis, caspase-3, is not activated in the process of pyroptosis, nor is caspase-8 or caspase-9[126, 130]. The cell lysis that occurs in pyroptosis is also very different from a major feature of apoptosis: i.e. an intact membrane with 'bleb' formation[126, 131].

Salmonella causes pyroptosis in bone marrow-derived macrophages within 1 hour of infection[132, 133] and SPI-1-encoded T3SS and flagellin are necessary for this process. Non-flagellated S. Typhimuriun[133, 134] or strains carrying mutations in *invA, invG, invJ, prgH, sipB, sipC, sipD* or *spaO,* cannot successfully induce pyroptosis[126, 130, 135-138], whereas strains deficient in some SPI-1 T3SS-translocated effectors, such as sipA and sptP, can affect pyroptosis induction[135]. Once *Salmonella* grows to the stationary phase, it reduces SPI-1 T3SS function and increases replication in macrophages without causing cell death[136]. However, *in vitro* studies have shown that the induction of cell death reappears 18-24 hours after macrophages are infected[124], and that the SPI-2 T3SS is responsible for this delayed pyroptosis[38, 138, 139].

1.7.3 Necroptosis in *Salmonella* infection

Necroptosis, another programmed cell death, may also occur in *Salmonella*-infected cells. *S.* Typhimurium induces a cell death, which is initiated by the TLR4/TRIF pathway and mediated by the RIP1/RIP3 complex. However, this form of cell death

appears to be detrimental for host defence[140]. How these different types of programmed cell death are regulated and orchestrated and what role they play in *Salmonella* infection is yet to be fully explained.

1.8 A key cytokine in *Salmonella* immunity: IFN-γ

In murine salmonellosis, interferon (IFN)-γ plays a critical role[141]. Similar phenomena have been reported in humans. Typhoid fever patients who have mutations in IFN-γ and IFN-γ receptor (IFN-γR) are difficult to treat with antibiotic therapy[142]. IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice, or mice with the cytokine neutralized by anti-IFN-g antibodies, are unable to survive infection with attenuated strain of *Salmonella*, whereas wild type B6 mice are able to survive and ultimately clear attenuated bacteria[141, 143-145]. In infected mice that are pre-treated with recombinant IFN-γ, the bacterial load is 70% lower than in control mice[141].

1.9 Key pathways of innate immunity leading to IFN-γ

1.9.1 NLRC4 inflammasome

NLR family CARD domain-containing protein 4 (NLRC4) forms one of the complexes known as an inflammasome. It senses cytosolic flagellin and activates the proteolytic enzyme caspase-1, also called interleukin (IL)-1β-converting enzyme (ICE)[146]. Caspase-1 associates with NLRC-4 via CARD-CARD interactions, and the adaptor protein ASC is considered a key component of the NLRC4 inflammasome[132-134]. Activated caspase-1 further converts pro-inflammatory cytokines IL-1β and IL-18 into their mature forms, both of which are crucial in inflammation[147]. IL-1b is involved in a variety of downstream immune responses[148-150] while IL-18 leads to the secretion of interferon-γ (IFN-γ) by natural killer (NK) cells and T cells. IL-18 is secreted by both DCs and macrophages that possess NLRC4[151, 152]. Also, the active form of caspase-1 drives pyroptosis, which may be important to host defence during salmonellosis, at least in the animal model[99, 153]. In addition to cytokine release and pyroptosis, NLRC4 in macrophages triggers actin polymerization to increase intracellular ROS, which facilitates killing of intracellular bacteria[154].

A number of studies have further explored the inflammasome pathway in *Salmonella* infection. Infection with flagellin-knockout *Salmonella* leads to impairment in caspase-1 activation and IL-b cleavage in *vitro*[133, 134], and/or IFN-γ secretion in *vivo*[152]. As previously stated, B6 mice infected with a *S.* Typhimurium strain that constantly expresses flagellin survived after infection, while NLRC4$^{-/-}$ mice exhibited a similar survival as wild type (WT) *Salmonella*-infected B6 mice[99]. Though an essential part of the NLRC4 inflammasome, ASC was shown to be dispensable for caspase-1 activation[132]. Very early research showed that mice with a caspase-1$^{-/-}$ mutation on the B10.RIII background were more resistant to orally inoculated *Salmonella* but responded the same as WT mice when the infection was given intraperitoneally (i.p.), suggesting a role for caspase-1 in the dissemination of *Salmonella* from the gastrointestinal tract[155]. Later studies of mice with a caspase-1$^{-/-}$ mutation on the B6 background could not reproduce this finding and showed increased susceptibility to oral infection with *Salmonella*[39, 156]. In addition, IL-18$^{-/-}$ mice showed a similar increase in susceptibility and bacterial load in the spleen, Peyers patches (PP) and mesenteric lymph nodes (MLN) as WT mice. However, caspase-1$^{-/-}$ and IL-18$^{-/-}$ mice were not protected against septic shock induced by i.p. injection of 10^8 CFU of live attenuated *S.* Typhimurium SL7207[156].

It is commonly considered that the cytosolic flagellin sensed by NLRC4 is delivered through T3SS, which is encoded by SPI-1 and SPI-2[133, 134]. Nevertheless, it has also been found that T3SS-deficient *S.* Typhimurium, heat-killed *S.* Typhimurium, or even purified flagellin, are capable of activating the NLRC4 pathway and inducing IFN-γ ex *vivo*[40] and in *vivo*[152]. At the same time, other heat-killed bacteria strains including *E. coli*, which is closely related to *Salmonella, H. pylori,* and *P. aeruginosa* [152] did not have this effect. In contrast, *Yersinia enterocolitica,* which does have flagella, drove IFN-γ release similar to *Salmonella*[152]. This data indicate that flagellin of *Salmonella* are able to enter host cell cytosol and activate NLRC4, however the mechanism of the entry is still unknown.

1.9.2 TLR5

After recognition of their ligands, TLRs typically induce the production of pro-inflammatory cytokines, chemokines, IFNs and co-stimulatory molecules.

Extracellular flagellin can be recognised by TLR5 that is expressed on the basolateral, but not apical, surface of polarised epithelia in gut[157]. This basolateral location may serve to reduce activation by non-invasive gut microbiota. Interestingly, TLR5 is not only activated by *Salmonella* that breaks through the epithelial barrier, but also by flagellin that was transcytosed across the epithelia. This transcytosis is also thought to be facilitated via SPI-2[158]. Activated TLR5 dimerises, recruits myeloid differentiation factor 88 (MyD88) and signals through the interleukin-1 receptor-associated kinase 4 (IRAK-4), which subsequently interacts with other members of the IRAK family, such as IRAK-1[159]. This recruitment leads to the activation of TNF receptor associated factors (TRAF), including TRAF6[160, 161]. TRAF6 further activates TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3 complexes[162], which activate mitogen-activated protein kinase (MAPK) (p38 and extracellular signal-regulated kinase (ERK)) and IkB kinase. IkB kinase is responsible for NF-kB activation, which results in the release of pro-inflammatory cytokines, such as neutrophil chemoattractant IL-8[163], and the activation of inducible nitric oxide synthase (iNOS). This cascade leads to peroxynitrite formation, cytotoxicity and the anti-bacterial function of macrophages[157, 164]. However, in murine salmonellosis, orally infected TLR5-null mice show decreased dissemination of the bacteria from the intestinal tract to the MLN, leading to increased survival[165].

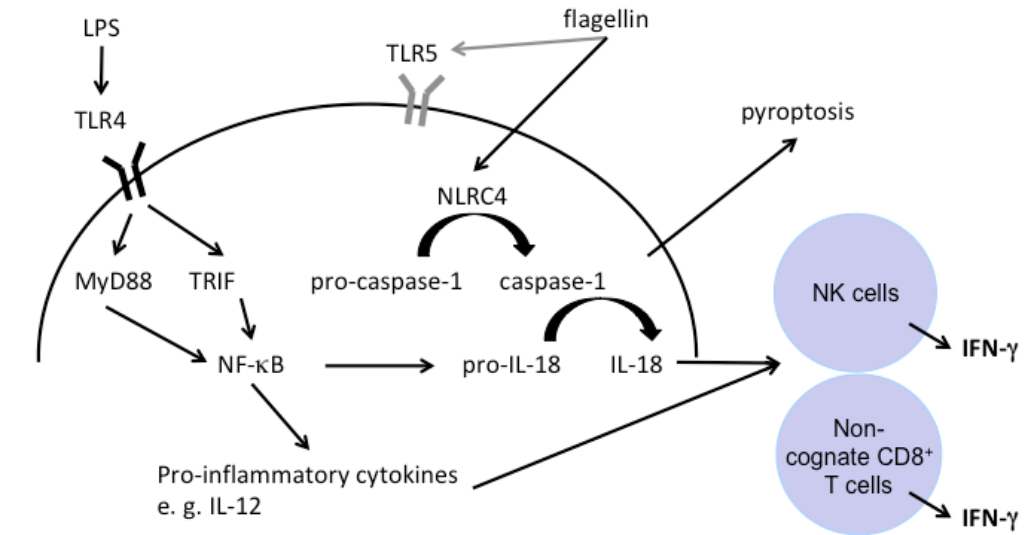

Figure 1-2: Key pathways of innate immunity leading to IFN-γ.

During Salmonella infection, NLRC4 inflammasome recognises intracellular flagellin and TLR4 senses extracellular LPS. Both activate reactions that lead to IFN-γrelease.[152, 166].

1.9.3 TLR4

TLR4, which senses LPS on Gram-negative bacteria, recruits MyD88 and TIRAP via TIR-TIR interactions, activates the canonical MyD88 signalling pathway[167], and induces cytokines including TNF-α, IL-6, IL-12p40 and IFN-γ[160, 161]. TLR4 is also able to signal through a MyD88-independent pathway that leads to Type I IFNs secretion. After associating with TRAM, TLR4 activates TIR-domain-containing adapter-inducing interferon-β (TRIF), and triggers TRAF3 and TRAF6[168-170]. TRAF3 is essential for the regulation of type I IFNs, which are able to stimulate natural killer (NK) cells and promote apoptosis in viral infection[171]. TRAF3 also regulates IL-10, an anti-inflammatory cytokine that inhibits IFN-γ[166]. The TRAF6 activation induces NF-κB and IFN-γ[159, 166].

It has been shown that intravenous (i.v.) infection with attenuated *Salmonella* leads to a much higher bacterial burden in the liver of TLR4$^{-/-}$ mice than WT mice, which indicates a role of TLR4 in murine salmonellosis. Further studies have been conducted to test which TLR4 pathway is responsible for bacterial control. MyD88$^{-/-}$ and TRIF$^{-/-}$ mice treated showed higher bacteria loads than WT mice during the time course, however the growth of the bacteria in MyD88$^{-/-}$ mice produced an experimental bacterial growth curve similar to that observed in TLR4$^{-/-}$ mice, while TRIF$^{-/-}$ mice demonstrated a relative slower rate of bacterial growth[172]. Significantly higher IFN-γ levels were found in TRIF$^{-/-}$ mice, possibly due to the absence of IL-10 induction through the TLR4/TRIF pathway[172].

In contrast, the downstream product of the TLR4/TRIF pathway, type I IFN, seems to have a detrimental effect on the outcome of *Salmonella* infection. It was recently found that mice deficient in the receptor for IFN-α and IFN-β (IFNAR-deficient (Ifnar1$^{-/-}$)), after either i.v. or i.p. infection with WT *S.* Typhimurium, have much lower bacterial counts in the spleen than B6 mice, and show prolonged survival[139]. It was suggested in this previous study that reduced programmed cell death occurs in Ifnar1$^{-/-}$ macrophages, leading to enhanced anti-bacterial function. Though this cell death is considered to be type I IFNs-induced and RIP1-RIP3 complex-mediated, RIP3$^{-/-}$ mice did not show any improvement in survival, compared to WT mice[140].

In infection with other Gram-negative bacteria, such as *Escherichia coli*, the TRIF pathway activates caspase-11 through type I IFNs and subsequently triggers the NLRP3 inflammasome. This mechanism seems to be dispensable in *S.* Typhimurium infection[173]. In *Yersinia enterocolitica* infection, TRIF-deficient macrophages are found to have impaired phagocytosis and thus reduced bactericidal function[174]. The mechanism through which the TRIF pathway contributes to bacterial control in *S.* Typhimurium infection is currently unclear.

1.10 Flagellar phase variant

As discussed previously, flagellin, the major structural component of the flagellar filament of *Salmonella*, activates several host defence mechanisms. Some serovars of *Salmonella*, such as *S.* Typhimurium, possess a mechanism, flagellar phase variation, to switch flagellin expression between FliC (flagellin phase 1/ H1) and FljB (flagellin phase 2/ H2), which is mediated by inversion of the promoter for FljB[175]. When the *fljBA* promoter is in the correct orientation for *fljBA* operon transcription, flagellin FljB is expressed, and also FljA, which is a transcriptional inhibitor of the *fliC* gene. Once the promoter is inverted, FljB or FljA cannot be expressed, thus flagellin FliC is produced[176]. The differential expression of flagellin leads to distinct antigenicities[177]. Though the molecular mechanism of flagellar phase variation is well understood, little is known of the biological role of this process. So far, it has only shown that FliC and FljB are equally capable of activating TLR5 and NF-κB[178], as well as in inducing IL-1β through NLRC4 pathway[179]. The role of the flagellar phase variation in *Salmonella* pathogenesis remains unclear and it is worth noting that some *Salmonella* serovars are monophasic[180].

1.11 Project Aims

Salmonella enterica is a Gram-negative intracellular pathogen, which can cause typhoid fever and non-typhoidal salmonellosis in humans and a range of veterinary infections. The emergence of antibiotic resistance has restricted treatment options, while currently available vaccines only provide partial protection against a single human serovar (*S.* Typhi), indicating a need for alternative treatment and preventative

options. *Salmonella* infection in mice is a well-established model for studying the host-pathogen interaction. Innate immunity provides the very early protection against *Salmonella* infection, but a better understanding of the early infection events may lead to new options for the treatment and prevention of *Salmonella* infection. This study aims to understand the very early death of DCs, which form a crucial part of innate immunity against *Salmonella* infection, and the role of I IFN-γ, an essential cytokine produced by a number of effector cells shortly after DC encounter with *Salmonella*.

Specific aims of the project are:

1. Clarify how *Salmonella* causes cell death in DCs (Chapter 3);
2. Study how IFN-γ, an essential cytokine that is produced as a consequence of DC/Salmonella interactions, contributes to *Salmonella* immunity (Chapter 4).

Chapter 2

Materials and Methods

2.1 Suppliers

Table 2-1 Locations for suppliers of reagents and equipment used in this study.

Supplier	Location
Applied Biosystems	Massachusetts, USA
BD Biosciences	California, USA
BecKanamycinan Coulter	California, USA
BioLegend	California, USA
BioRad	California, USA
Chem Supply	South Australia, Australia
Corning	Massachusetts, USA
eBioscience	California, USA
Eppendorf	Hamburg, Germany
Fisher Biotech	Western Australia, Australia
Geneworks	South Australia, Australia
Gibco, Invitrogen Coorperation	Massachusetts, USA
Invitrogen	Massachusetts, USA
Merck	Massachusetts, USA
MilliPore	Massachusetts, USA
Miltenyi Biotech	Bergisch Gladbach, Germany
New England Biolabs	Maryland, USA
Oxoid	Hampshire, UK
Promega	Wisconsin, USA
Qiagen	Victoria, Australia
R&D Systems	Minnesota, USA
Roche	Mannheim, Germany
Sarstedt	Nümbrecht, Germany
Seward	West Sussex, UK
Siemens	New York, USA
Sigma-Aldrich	Missouri, USA
Thermo Fisher Scientific	Massachusetts, USA

2.2 Bacteria

2.2.1 Bacterial strains and plasmids

Table 2-2 Bacterial strains and plasmids used in this study.

Bacteria/strains	Antibiotic resistance	Characteristics	Source/Reference
***Salmonella enterica serovar* Typhimurium**			
SL1344	Streptomycin	Wild type strain	G. Dougan[1]
SL1344-GFP	Streptomycin, Ampicillin	Wild type strain carries GFP on plasmid (pGF3)	Fu Guo[2]
SL1344 *ΔinvA*	Streptomycin, Kanamycin	SPI-I effector deficient	R. Curtiss III[3]
SL1344 *ΔssaR*	Streptomycin	SPI-II effector deficient	R.K.R. Mantena[4]
SL1344 *ΔfliCΔfljB*	Streptomycin	Non-flagellated	R. Curtiss III [3]
SL1344 *ΔinvAΔfliCΔfljB*	Streptomycin, Kanamycin	SPI-I effector deficient and non-flagellated	This study
SL1344 *ΔfliC*	Streptomycin	Flagellated, no phase 1	Hanwei Cao[4]
SL1344 *ΔfljB*	Streptomycin	Flagellated, no phase 2	R. Curtiss III[3]
SL1344 *ΔfliCΔfljB* pLS408	Streptomycin, Ampicillin	Non-flagellated strain complemented with FliC	P. Whitney [4]
BRD509	Streptomycin	*ΔaroA* mutant of SL1344	G. Dougan[1]
Plasmids			
pLS408	Ampicillin	pUC19 expressing H1-d gene from *S. muenchen*	[181]
pGF3	Ampicillin	pTET(tac4) containing GFPmut3b encoding sequence instead of c fragment of tetanus toxin	Fu Guo[2]

[1]Wellcome Trust Sanger Institute, Cambridge, UK

[2]College of Materials Science and Engineering, Beijing University of Technology, China

[3]Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida, USA

[4]Department of Microbiology and Immunology, The University of Melbourne, at Peter Doherty Institute of Infection and Immunity, Victoria, Australia

2.2.2 Growth conditions for *S.* Typhimurium

For growth in liquid cultures, *S.* Typhimurium strains were cultured in Luria broth (LB) static at 37°C overnight for infection experiments, or shaking at 180rpm at 37°C overnight for generating heat-killed bacteria or for phage transduction. To obtain single colonies or colony counts, the bacteria were grown on LB agar plates at 37°C overnight. Antibiotics were supplemented accordingly at the following concentrations: 25μg/ml streptomycin, 50μg/ml kanamycin, and 100μg/ml ampicillin.

2.2.3 Preparation of bacteria for infection

S. Typhimurium cultures, grown statically at 37°C overnight in LB broth, were harvested and washed in sterile phosphate-buffered saline (PBS). The optical density at 600nm (OD_{600}) of the bacterial suspension was measured to estimate cell number. Bacteria were then diluted in PBS to the required concentration for infection experiments.

2.2.4 Preparation of heat-killed *S.* Typhimurium

S. Typhimurium were cultured as described in Section 2.2.2 in shaking condition and harvested. Bacteria were then washed in sterile PBS twice, and the bacterial concentration was estimated using OD_{600}. An aliquot of the bacterial suspension was plated on LB agar plates to accurately determine the bacterial number. The remaining bacteria were heat-inactivated at 65°C for 1 hour. After heat treatment, an aliquot was plated on LB agar plate and incubated overnight at 37°C to confirm the heat-kill was effective and the heat-killed bacteria were adjusted to a concentration of 5×10^8cfu/ml and stored at -20°C.

2.2.5 Motility test

To determine motility, bacteria were inoculated on soft LB agar (0.3%) plates and incubated at 37°C overnight. Flagellated (motile) bacteria are able to spread across the surface of the soft agar, whereas non-flagellated bacteria remain at the spot of inoculation due to the lack of motility.

2.2.6 P22 phage-mediated transduction

P22 phage transduction is a method to transfer genomic mutations from one *S.* Typhimurium strain to another. 48Kb bacterial chromosomal DNA can be packaged into the P22 phage after the bacterium is infected, and when the phage infects a new bacterium, this chromosomal DNA can be injected into the new bacteria and recombine into the chromosome. To generate donor phage lysate, donor bacterial strain was cultured as described in 2.2.2 and sub-cultured for approximately 3 hours till mid-log phase. For 1.5ml bacterial culture, 50µl P22 (HT105/2) int^- phage lysate, which is the phage lysate of wild type *S.* Typhimurium SL1344, was added and incubated shaking at 180rpm at 37°C overnight. 50µl chloroform was added to the phage lysate and the lysate was incubated shaking at 180rpm at 37°C for 20min to lyse the remaining bacteria. Cells were pelleted by centrifuging at 2800 × *g* for 2min, and the supernatant was collected into a new microfuge tube. The donor lysate was again mixed with 30µl chloroform and incubated shaking at 180rpm at 37°C for 3h. An aliquot of lysate was plated out on LB agar plates with appropriate antibiotics to confirm that the lysate contained no viable donor bacteria. Phage lysate was stored at 4°C. Recipient bacteria strain was cultured as described in 2.2.2 and sub-cultured. Bacteria were harvested in approximately 3 hours after sub-culture when in mid-log phase. Serial dilutions of donor phage lysate were mixed with equal volumes of recipient bacteria culture and incubated shaking at 180rpm at 37°C for 20-30min. The recipient phage lysate mixes were plated out on LB agar plates with appropriate antibiotics.

2.3 *In vitro* and *ex vivo* work

2.3.1 Generating bone marrow derived GM-CSF DCs

Bone marrow was flushed out from femurs bones of various strains of mice post mortem with RPMI-1640 (Invitrogen). Bone marrow was pipetted to form a consistent cell suspension. Red blood cells (RBCs) were lysed using TAC buffer (17mM tris, 140mM ammonium chloride in distilled water) for 10min at RT and the cells were filtered through a 100µm cell strainer (BD Falcon) to remove any cell debris and clumps. After washing in RPMI-1640, an aliquot of cell suspension was removed and diluted in 0.4% trypan blue (Sigma) to determine the cell count using a

haemocytometer. The remaining cells were centrifuged (350 × g at 4°C for 5 min) and resuspended in RPMI-10 (RPMI 1640 + 10% foetal calf serum (FCS)+ 2mM L-glutamine (Invitrogen) +100 Units/ml penicillin and 100 μg/ml streptomycin (Invitrogen)) with 5% GM-CSF conditioned media harvested from supernatants of Ag8653 cells transfected with the GM-CSF gene, as described in section 2.3.4. Cells were cultured in 6-well tissue culture plates at 37°C with 5% CO_2 for at least 6 days before use.

2.3.2 Generating bone marrow-derived Flt3L DCs

Bone marrow cell suspension was obtained as described before for GM-CSF DCs (2.3.1). After RBC lysis, an aliquot of cell suspension was used to determine the cell concentration and the rest was centrifuged and resuspended in RPMI-10 with 200ng/ml human recombinant Flt3L (a gift from Dr. Yifan Zhan (Walter and Eliza Hall Institute for Medical Research, Australia)). Cells were cultured in 6-well tissue culture plates at 37°C with 5% CO_2 for 9 days before use.

2.3.3 Generating bone marrow derived macrophages

Bone marrow cell suspension was obtained as the method described in 2.3.1 and resuspended in RPMI-10 with 30% M-CSF conditioned media derived from supernatants of L929 cells. Cells were cultured in tissue culture dishes at 37°C with 5% CO_2 for at least 6 days before use.

2.3.4 Culturing Ag8653 cells and supernatant collection

Ag8653 cells are derived from murine myeloma, transfected with GM-CSF cDNA[182] and the cells were cultured with RPMI-10 in cells culture flasks 37°C with 5% CO_2. GM-CSF-containing supernatant was collected and centrifuged (350× *g* for 5min) to remove the remaining cells. Supernatant was then stored at -80°C.

2.3.5 Cell infection with *S.* Typhimurium

Various strains of *S.* Typhimurium were prepared as described in 2.2.3. Bone marrow derived DCs or macrophages were harvested and resuspended in the appropriate

culturing media without antibiotics. Cells were labelled with CellTrace Violet (Invitrogen) according to the manufacturer's instructions and counted by using trypan blue. Briefly, cells were labelled at 37°C for 20min and washed 3 times in culturing media with no antibiotics. Labelled cells were seeded at 10^5 cells per well in 12-well tissue culture plates. Bacteria were added to DCs or macrophages at various multiplicities of infection (MOI), centrifuged (350 × g for 5 min), and incubated for 1h at 37°C with 5% CO_2. After the initial 1 hour period, the media was replaced with RPMI-10 supplemented with 40µl/ml gentamicin, and cells were further incubated for 1h at 37°C with 5% CO_2. Cells were removed from the wells by incubating with 0.4% lidocaine and 5mM EDTA in PBS for 5min, washed with PBS, then analysed using flow cytometry or microscopy. For viable bacterial counts, cells were washed 3 times with sterile PBS and lysed with 0.5% (v/v) triton X-100 for 15min at room temperature (RT) to release intracellular bacteria, which was then plated on LB agar plates with selective antibiotics and cultured overnight at 37°C.

2.3.6 Supernatant collection

Cells were infected as described in 2.3.5 and before cells were removed from wells for further study, the supernatant was removed carefully from the wells without disturbing the cells. The supernatant was then filtered through syringe filters with pore size of 0.4µm to remove any carryover cells or bacteria. Aliquots of the supernatant were stored at -20°C.

2.4 Animal work

2.4.1 Mice strains

Table 2-3 Stains of mice used in this study.

Name	Source	Reference
C57BL/6	UoM	
$TLR4^{0/0}$ *	UoM	[183]
$MyD88^{-/-}$	UoM	[184]
$TRIF^{-/-}$	UoM	[169]
$ICE^{-/-}$ ($Caspase\text{-}1^{-/-}Caspase\text{-}11^{-/-}$)	UoM	[185]
$IL\text{-}18^{-/-}$	UoM	[186]
$IFN\text{-}\gamma^{-/-}$	UoM	[187]

IPAF$^{-/-}$ (NLRC4$^{-/-}$)	UoM	[132]

UoM: The University of Melbourne

* These TLR4$^{0/0}$ mice carry the point mutation originally observed in C3H/HeJ mice and were backcrossed at least 13 generations onto the C57BL/6 mouse line.

2.4.2 Animal husbandry

All animals listed in Table 2-3 were bred in the specific pathogen free (SPF) facility at the Department of Microbiology and Immunology, The University of Melbourne. Mice were supplied with sterile food and water *ad libitum* and euthanised by CO_2 asphyxiation.

2.4.3 Animal ethics

All animal experiments were approved by Animal Ethics Committee at the University of Melbourne.

2.4.4 Infection

All mice in this study were infected intravenously. Live bacteria, *S.* Typhimurium SL1344 or BRD509, were grown and prepared as described in sections 2.2.2 and 2.2.3 and adjusted to desired concentration in sterile PBS. Heat-killed bacteria were prepared as described in section 2.2.4. For every infection, an aliquot of live bacteria was diluted and plated out on LB agar plates with selective antibiotics and cultured at 37^{o}C overnight for a more accurate bacterial count of the inoculum.

2.4.5 Intravenous injections

Mice were warmed near a heat source prior to injections to dilate the tail veins. Mice were then individually moved to a restrainer and 200µl of inoculum or PBS was injected into the tail vein using 27G needles.

2.4.6 Monitoring infected mice

For wild type *S.* Typhimurium SL1344 infection, mice were visually inspected twice a day for health conditions and weighed daily. For attenuated *S.* Typhimurium BRD509 infection, mice were visually monitored daily from day 0 to day 3 post

infection, twice a day from day 4 to day 8 post infection, and three times a week afterwards. And mice were weighed weekly.

2.4.7 Intraperitoneal injections

In this study, intraperitoneal injections were used to deliver neutralising antibodies. The mouse was restrained by the scruff of the neck and turned over to expose the abdominal region to be injected. An ethanol wipe was used to clean the injection site and 200µl of antibody or PBS were injected using 25G needles.

2.4.8 Preparation of splenic single-cell suspension

At certain time points post-infection, mice were euthanised by CO_2 asphyxiation. The mouse was sprayed with ethanol to make a sterilised work area. The spleen was collected into RPMI-1640 with 2.5% FCS and 1mM EDTA and kept on ice. The spleen was pushed through a 100µm cell strainer that dissociated cells into a single-cell suspension. Cells were centrifuged at 350 × g, 4°C for 5min and re-suspended in TAC buffer for 10min at RT to lyse red blood cells. Cells were centrifuged at 350 × g, 4°C for 5min and re-suspended in appropriate buffers for following procedures.

2.4.9 Measuring bacterial load in infected spleen and liver

At certain time points post-infection, mice were euthanised by CO_2 asphyxiation. The mouse was sprayed with ethanol to make a sterilised work area. The spleen and liver were collected into sterile PBS and kept on ice. Organs were weighed before transferred into sterile Stomacher bags (Sarstedt) with 5ml of PBS. Samples were put in a Stomacher 80 homogeniser (Seward Medical) to be homogenised and then diluted serially. Different dilutions of homogenates were plated on LB agar plates with appropriate antibiotics and incubated at 37°C overnight. The bacterial load per organ was calculated according to the bacterial counts on plates.

2.4.10 Serum collection

Blood samples were collected by cardiac puncture immediately after the mouse was euthanised and kept at RT for 3 hours to clot. Blood samples were centrifuged in a

microfuge at 1400 × g for 10min. Serum was collected, with care taken not to disturb the pelleted cells, and stored at -20°C.

2.4.11 IFN-γ secretion assay

Mice were injected intravenously as described in section 2.4.4 with different strains of heat-killed *S.* Typhimurium. Heat-killed bacteria were prepared as described in section 2.2.4. Two hours after injection, the mouse was euthanised by CO_2 asphyxiation and the spleen was harvested. Single-cell suspensions were prepared as described in section 2.4.6. Cells were then washed with PBS with 0.5% BSA and IFN-γ secretion was measured using the Mouse IFN-γ Secretion Assay Detection Kit (Miltenyi Biotec) as per manufacturer's instructions. Briefly, cells were incubated with IFN-γ capture antibody on ice for 5 minutes and then transferred into a 10ml tube, topped up with RPMI+2.5% FCS, and incubated with rotation for 45min at 37°C. Cells were washed and stained with IFN-γ detection antibody PE and other surface markers (listed in table 2-4). Cells were washed and analysed by flow cytometry.

2.4.12 Cytometric Bead Array (CBA)

The level of inflammatory cytokines, such as IFN-γ, TNF-α, MCP-1, IL-6, IL-12p70 and IL-10, were determined by using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences) according to manufacturer's instructions. Briefly, samples were incubated with cytokine-specific capture beads and stained with PE detection reagent for 2h at RT in dark. Flow Cytometry was used to collect raw data and data was analysed by using the FCAP Array software (BD Biosciences).

2.5 Flow Cytometry

2.5.1 Staining and data collection

Cells were treated with Fc Block™ (purified rat anti-mouse CD16/CD32, BD Biosciences) in FACS buffer (PBS with 0.1% (w/v) BSA and 5mM EDTA) for 15min on ice to reduce non-specific antibody binding. Cells were then washed with FACS buffer and stained with various surface antibodies (listed in table 2-4) for 30-60min on ice. Aliquots of cells were not stained or stained with single antibody as unstained

control or compensation controls, respectively. After staining, cells were washed twice in FACS buffer. Cells were re-suspended in FACS buffer with 2 μg/ml propidium iodide (PI) and calibration beads (Sphero^{TM} Blank Calibration Particles, 6.0–6.4μm, BD Biosciences). If cells were stained for Annexin V (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen), cells were washed in cold PBS after staining for surface markers. Cells were then re-suspended in 1× Binding Buffer and stained with Annexin V and PI for 15min at RT in the dark. 400μl of 1× Binding Buffer was then added to each sample after the staining. Cells were analysed by BD LSRII flow cytometer or BD LSRFortessa (BD Biosciences). Data was analysed using the FlowJo software (FlowJo, LLC).

2.5.2 Antibodies

Table 2-4 Antibodies used in this study.

Antigen	Clone	Conjugate	Source
CD11b	M1/70	FITC, APC, APC-Cy7	BD Pharmingen
CD11c	HL3	FITC, APC	BD Pharmingen
CD19	1D3	FITC, PE-Cy7, AlexaFluor700, PerCP-Cy5.5	eBioscience BD Pharmingen
CD3e	17A2 145-2C11	V450, AlexaFluor700, FITC, PE	BD Horizon BD Pharmingen eBioscience
CD4	L3T4 (RM4-4) GK1.5	FITC, PE-Cy7, APC-Cy7	BD Pharmingen
CD62L	MEL-14	FITC, PE-Cy7	eBioscience BD Pharmingen
CD8a	53-6.7	BV711, PE-Cy7, AlexaFluor700	BD Horizon BD Pharmingen
F4/80	BM8	FITC, APC	eBioscience
IFN-γ	XMG1.2	FITC, PE	BD Pharmingen
Ly6C	AL-21	V450, BV605	BD Horizon
Ly6G	1A8	PE-Cy7	BD Pharmingen
MHC-II	AF6-120.1	eFluor450, PE	eBioscience BD Pharmingen
NK1.1	PK136	PE	BD Pharmingen
SiglecH	eBio440c	PE	BioLegend
Strepavidin	NA	PE	

2.6 Microscopy of infected dendritic cells

Bone marrow-derived dendritic cells were cultured as described in section 2.3.1 and infected as described in section 2.3.5 Round coverslips were placed into 24-well tissue culture plates and coated with anti-MHC-II N22 antibody for 2 hours at 37^{o}C. Coverslips were washed with PBS with 2% FCS, dried, and transferred into new wells. 2×10^{5} DCs in 20µl culture medium were added on each coverslip and incubated 10min at RT and then 20min at 37 ^{o}C. 500µl of culture medium was supplied to each well and cells were incubated for 2h at 37 ^{o}C to allow dendritic cells to attach to the coverslips. After washing with PBS, cells were treated with 4% paraformaldehyde (PFA) in PBS for 30min at RT to be fixed, and permeabilised with 0.3% triton X-100 in PBS for 5min at RT. Cells were blocked with 0.1% triton X-100 with 10% normal goat serum in PBS for 1 hour at RT. Cells were stained with primary antibody in blocking buffer at 4 ^{o}C overnight and secondary antibody in blocking buffer for 1 hour at RT in dark. Phalloidin was added at last to stain nuclei for 5min at RT in dark. Coverslips were then transferred onto microscopy slides and mounted with ProLong® Gold Antifade Mountant (Invitrogen). Slides were kept at RT for 24 hours to dry and can be stored at 4 ^{o}C in dark for months. Zeiss LSM 700 Confocal (Zeiss) was used to take images of the cells and ImageJ (NIH) was used to process the data.

2.7 Statistical analysis

GraphPad Prism 6 MacIntosh (GraphPad Prism Software, Inc.) was used for generating figures and performing statistical analyses. To compare two groups of data, the two-tailed unpaired *t*-test was used. To compare more than two groups of data with one variable, the one-way ANOVA with Tukey post-test was used. To compare data groups with two variables, the two-way ANOVA with Tukey post-test was used. Data on bacterial load were log-transformed before statistical analysis was performed. P values below 0.05 were considered significant.

Chapter 3

The early death of Dendritic cells in *Salmonella* Typhimurium infection

3.1 Introduction

Wild type *S.* Typhimurium SL1344 causes lethal infection in C57BL/6 (B6) mice within 7-10 days, following intravenous (i.v.) or oral infection, and the animals show signs of typhoid fever including weight loss, hepatomegaly, splenomegaly and high bacterial load in the liver and spleen following infection[67]. The net growth of SL1344 in B6 mice increases approximately 10-fold per day in the liver and spleen, after i.v. administration of as few as 10 bacteria[71, 188]. The bacterium resides and replicates within various cells including macrophages and dendritic cells[189, 190] but the failure to control the infection suggests that the interaction between the bacterium and the adaptive immune system, over a time frame where e.g. influenza virus is controlled by $CD4^+$ and $CD8^+$ T cells[191], is perturbed. The fulminant infection caused by SL1344 infection suggests a failure of innate immunity, and/or the failure to trigger protective T cell-mediated immune responses. This study tests the hypothesis that dendritic cells (DCs), as the key cellular link between innate immunity and adaptive immunity[85-95], are affected by *Salmonella* Typhimurium infection, impacting innate and adaptive immune responses.

As one of the first cells to interact with a pathogen during infection, DCs are crucial in the development of both innate and adaptive immune responses[85, 86, 94, 190, 192, 193], and the host defence could be impaired if *Salmonella* limits the function of DCs during the early stages of infection. Previous studies suggest that *Salmonella* is able to cause various forms of programmed cell death, including apoptosis, pyroptosis and necroptosis in a variety of cells[114-116, 126-128, 140]. While *Salmonella* regulates host cell death through secretion of type 3 secretion system effectors[102, 107, 108, 112, 113, 194, 195], the bacterium might also interfere with DC functioning by down-regulating expression of flagellin[99-101], regulating phagolysosomal fusion, delaying vacuole acidification and forming a complex known as the *Salmonella*-containing vacuole (SCV) to promote survival inside DCs[38, 39 102-104]. Having suppressed intrinsic anti-microbial responses, *Salmonella* can also manipulate the expression of MHC molecules and antigen presentation on the DC cell surface, thereby reducing targeting by effector T cell responses[112, 113]. However, much of the data concerning DCs has been generated *in vitro* and the impact of the *Salmonella*-DC interaction in the whole animal remains unresolved.

Overall the aims of the studies presented in this Chapter are:

a. to establish the system for infecting BMDCs with *S.* Typhimurium *in vitro*;

b. to investigate bacterial factors that contribute to the early and quick death in BMDCs;

c. to understand what factors from host cells are involved in the mechanisms of the early DC death.

3.2 Results

3.2.1 DCs are significantly reduced after *Salmonella* infection *in vivo*

To determine whether infection of mice might lead to either DC death or migration, mice were infected with *Salmonella* Typhimurium SL1344 and the numbers of B6 DCs enumerated by flow cytometry. Two bacterial doses were injected and the presence of DCs estimated at three time points following infection. Mice were i.v. infected with 10^4 cfu of *S.* Typhimurium SL1344 for 24h (Fig.3-1B-D), 48h (Fig.3-1E-G) or 200 cfu for 5 days (Fig.3-1H-J). Within the total splenic DC populations ($CD3^-CD19^-CD11c^+$), conventional DCs (cDCs, defined as $CD11c^{high}MHC\text{-}II^+$) were significantly reduced after i.v. infection with 10^4 S. Typhimurium. The number of plasmacytoid DCs (pDCs) (defined as $CD11c^+CD11b^-MHC\text{-}II^+SiglecH^+$) were reduced within 2 days of infection but the decrease in DC numbers was more marked by day 5. In contrast, monocyte-derived DCs (moDCs) (defined as $CD11c^{int}CD11b^{high}MHC\text{-}II^+Ly6G^-Ly6C^+$) were unchanged 24 hours after infection, but started to expand 2 days after infection. However, by 5 days post-infection the number of moDCs decreased to a level lower than naïve mice (Fig.3-1). This reduction of DCs could be explained in several ways – that DCs 1) down-regulate surface markers, 2) are mobilised to other sites, or 3) were killed and removed during early *Salmonella* infection. In order to investigate this phenomenon further, an *in vitro* system was developed to study DC-*Salmonella* interactions.

3.2.2 *in vitro* bone marrow derived dendritic cells (BMDC) infection model

Bone marrow cells were removed post mortem, and cultured *in vitro* for 6 days with GM-CSF, which stimulates hematopoietic bone marrow precursors to differentiate into DCs that resemble moDCs[196-199]. After culturing, BMDCs were harvested and infected with *S.* Typhimurium SL13344-GFP at various multiplicities of infection (MOI). Upon receiving danger signals, such as bacterial components, e.g. *Salmonella* LPS, DC maturation is triggered and maturation markers, such as MHC-II, CD40 and CD86, are up-regulated[200-203]. Only after maturation, DCs start to initiate immune responses, such as the production of cytokines and antigen presentation for priming T cell responses. For most of the experiments, DCs were examined by flow cytometry and defined by the expression of CD11c and CD11b. DCs associated with *Salmonella*

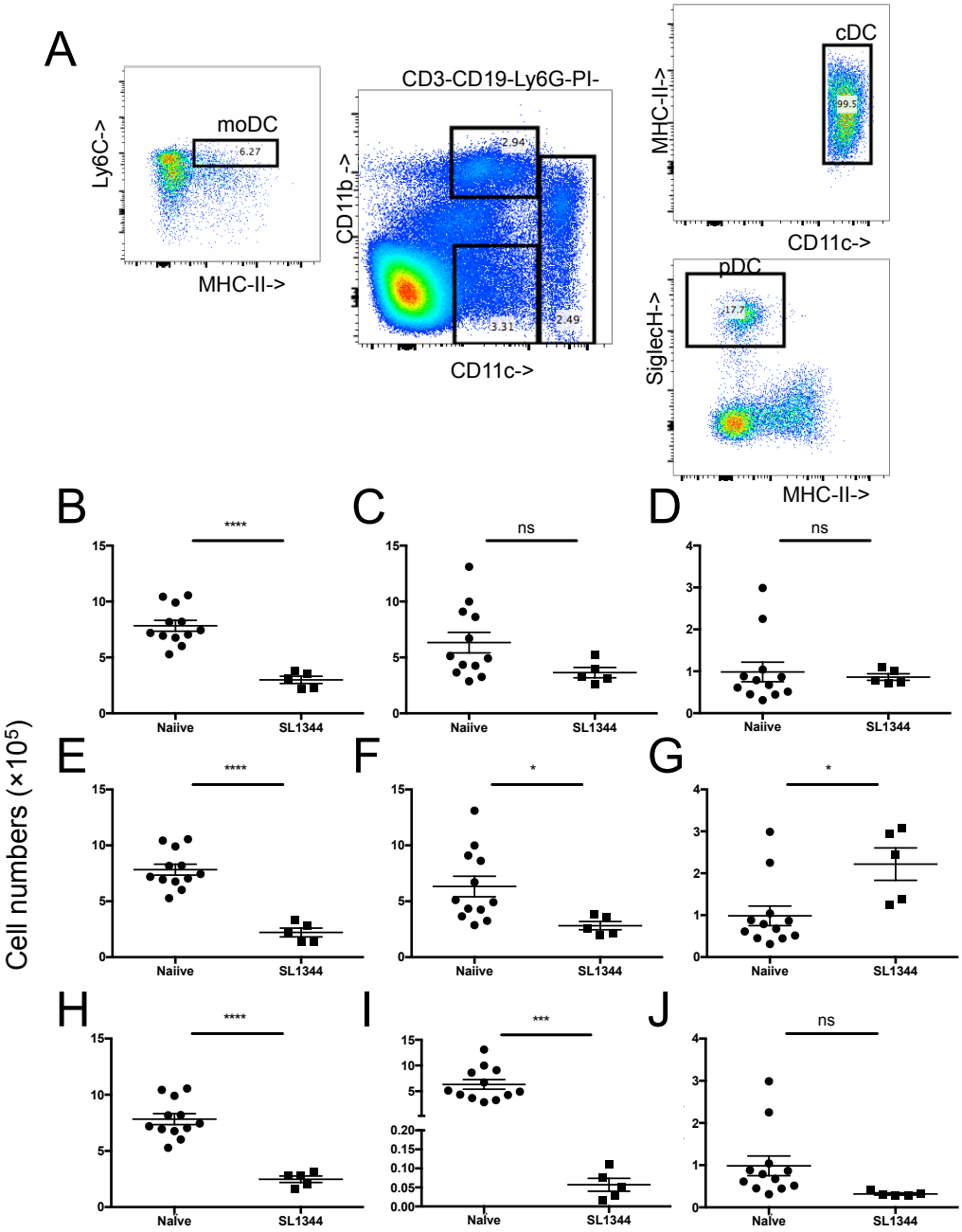

Figure3-1: Splenic DC numbers are changed in DC subsets after Salmonella infection.

*Mice were i.v. infected with 10^4 cfu of STM SL1344 for 24h (B-D) 48h (E-G) or 200 cfu for 5 days (H-J). At set time points, splenocytes were harvested from infected (square) and naïve mice (circle) and stained with fluorescent conjugated monoclonal antibodies for various markers of DC subsets and examined by flow cytometre. DC subsets were gated upon the marker expressions (A). Conventional DCs (cDC) were defined as $CD11c^{high}MHC\text{-}II^{+}$ (B,E,H), plasmacytoid DCs (pDC) were defined as $CD11c^{+}CD11b^{-}MHC\text{-}II^{+}SiglecH^{+}$ (C,F,I), monocyte derived DCs (moDC) were defined as $CD11c^{int}CD11b^{high}MHC\text{-}II^{+}Ly6G^{-}Ly6C^{+}$ (D,G,J). Symbols represent data from individual animals, shown are total number of each DC subset per spleen. Data for naïve group were pooled from all three time points. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM). Unpaired t tests were used for statistical analyses. *p<0.05 ***p<0.001 ****p<0.0001. Data are representative of at least 2 independent experiment.*

were detected using GFP, ie. GFP^+ (Fig.3.2A).

Confocal microscopy was performed to confirm that *S.* Typhimurium SL1344-GFP was present within B6 BMDCs, either through active invasion mediated by *S.* Typhimurium SPI-1[133, 134] or after phagocytosis[85, 86] by DCs (Fig.3-2B). BMDCs were infected with *S.* Typhimurium SL1344-GFP (green) at MOI 10 for 2 hours and 2×10^5 cells were transferred onto microscopy coverslips that were coated with a-MHC-II N22. Cells were then stained with α-MHC-II N22 (red) to show the cell membranes and DAPI (blue) to show nucleus. Coverslips were transferred onto microscopy slides and observed under confocal. A Z-stack, a series of continuous layers of the cell acquired by the scanning lasers and compressed in order to create a 3D image of the cell to confirm that the bacteria, shown in green, was inside of the DC. Fig.3-2B presents one of the layers where the structure of DC and the *Salmonella* bacteria is clearest and most intact.

To provide a positive control of death of infected DCs, staurosporine was used to trigger apoptosis[204] in the BMDCs, and CellTrace Violet (CTV) and PI stain to differentiate viable and non-viable cells. After 2h of treatment with staurosporine, the number of cells in the CTV^+PI^- population is significantly reduced (Fig.3-2C). propidium iodide (PI), binds to DNA or RNA and it is not able to permeate the cell membrane and is therefore excluded from the nuclei of living cells[205]. CTV diffuses through cell membrane and covalently binds to intracellular amines; CTV titration is usually used to track cell proliferation[206]. As cell membrane rupture or as blebbing occurs during the process of programmed death[125, 126, 131], the loss of CTV appears to indicate early cell death through membrane losses. In subsequent experiments, the CTV^+PI^- population (defined by the gating as shown in Fig.3-2D) was defined as viable cells and the cells that were excluded from the gate were defined as dying/dead cells. BMDCs generated via two methods were compared after infection with *S.* Typhimurium SL1344, and no significant difference was observed in the staining profile or the level of death between Flt3L-BMDC and GM-CSF-BMDC following *Salmonella* infection (Fig.3-2D). Thus, given the cost and ease of production advantages shown by the GM-CSF-BMDCs, these cells were used for subsequent experiments.

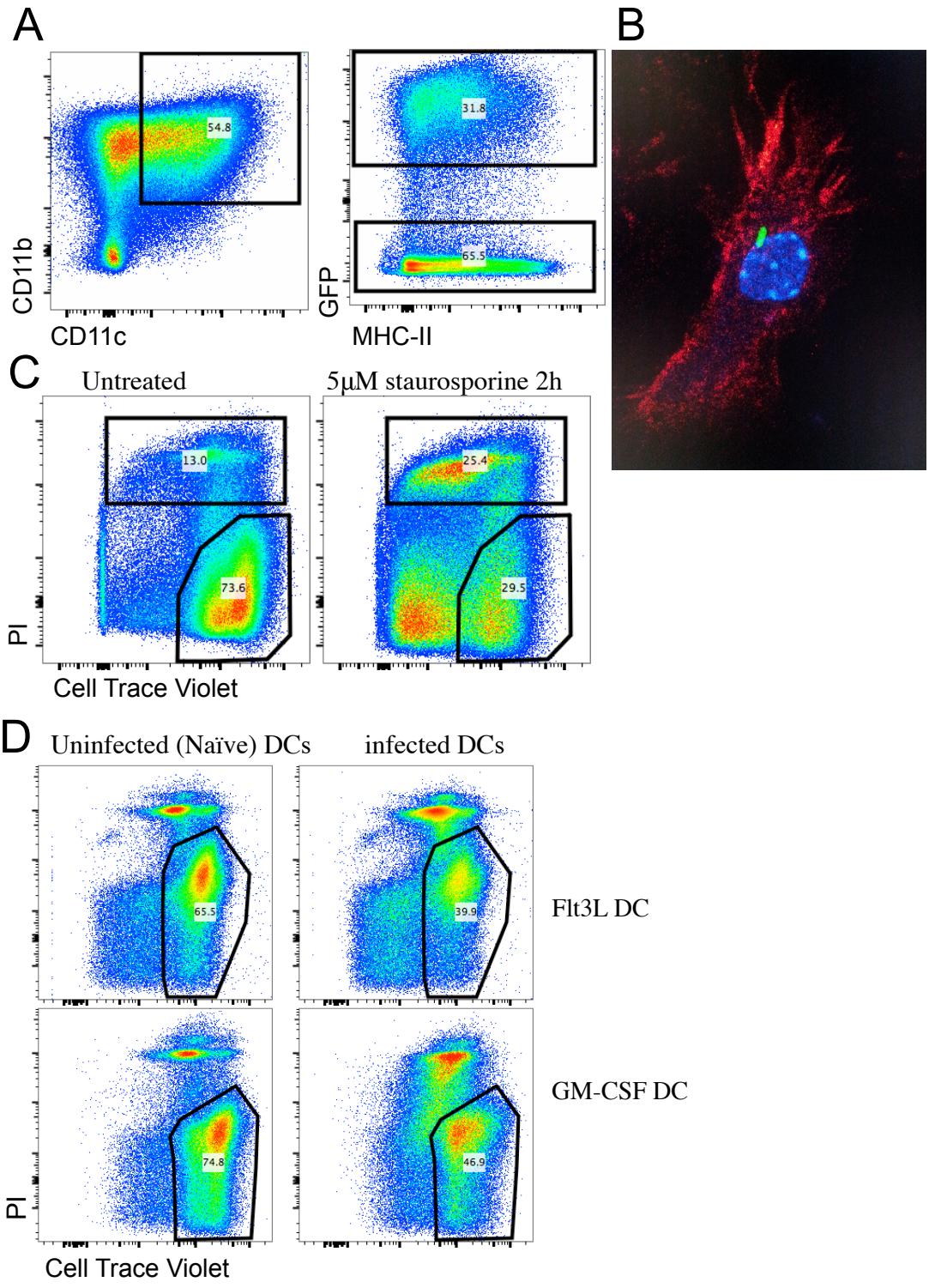

Figure 3-2: Establish the in vitro BMDC infection system.

Bone marrow cells from femurs were harvested and cultured with GM-CSF or Flt3L to generate BMDCs. After harvesting the DCs, cells were infected with STM SL1344-GFP at MOI 10 or 20 (A, B, D) or treated with 5uM staurosporine (C) for 2 hours. Cells were then stained with fluorescent conjugated monoclonal antibodies for DC markers or death markers and examined by flow cytometre (A,C,D) or transferred to microscopy slides that were coated with α-MHC-II antibody, and stained with α-MHC-II (red), and DAPI to show nucleus (blue) and observed under confocal (B).

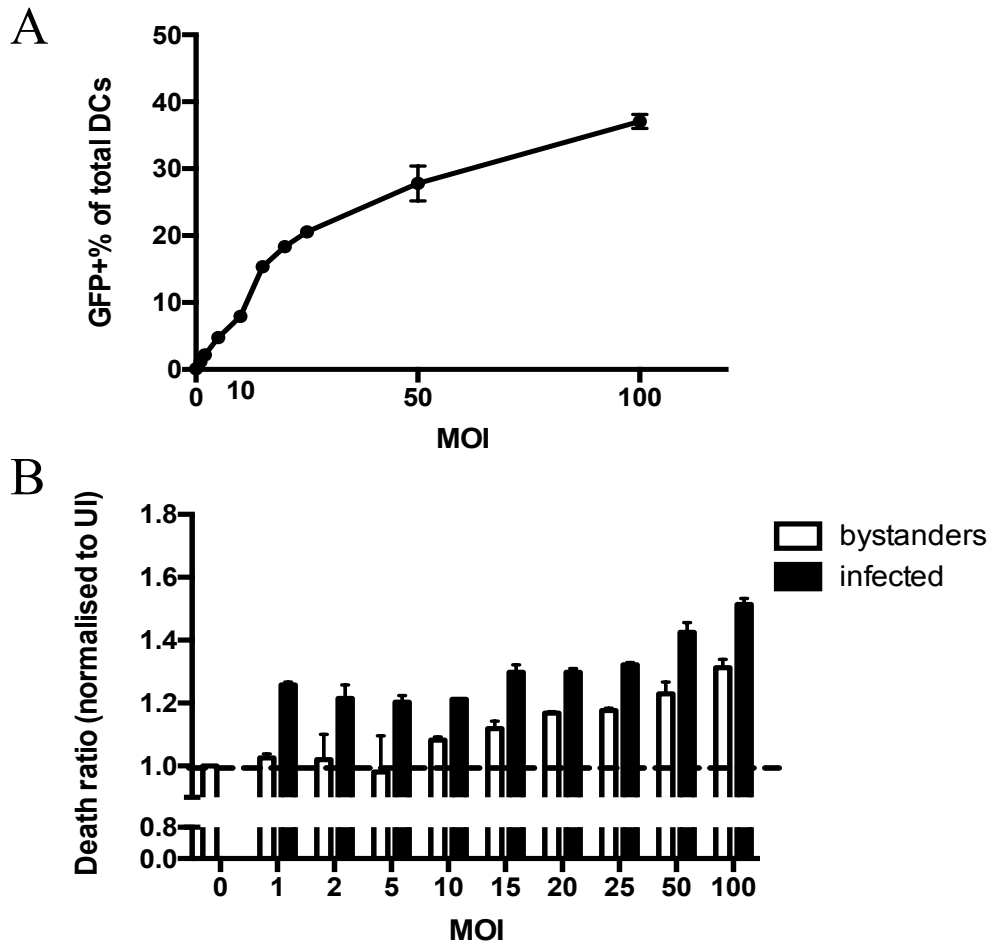

Figure 3-3 : ***Cell death occurs in both infected and bystander DCs in vitro.***

BMDCs were infected **:** *with GFP-expressing SL1344 with an increasing multiplicity of infection (MOI) for 2h. The GFP expression separates cells into two populations (Fig. 3.2A) and the infection ratio was calculated (A). Analysis on cell death was further done on both of the GFP^+ and GFP^- populations. Shown are the loss of viability in infected (GFP^+) BMDCs (black bars) and bystander (GFP^-) BMDCs (white bars) from the same wells. The CTV^+PI^- population (defined by the gating as shown in Fig.3-2D) was defined as viable cells and the cells that were excluded from the gate were defined as dying/dead cells. The percentage of dying/dead cells in a population was defined as death ratio and the death ratio of UI (MOI 0) was defined as 1. The data of other groups were normalised to the UI. Two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical analyses. In infected groups (black bars), $p<0.001$ for MOI 1-10 and $p<0.0001$ for MOI 15-100 . In bystanders groups (white bars), MOI 1-15 showed no significance, $p<0.01$ for MOI 20-25, $p<0.001$ for MOI 50, and $p<0.0001$ for MOI 100. (B) Each column represent 2 technical replicates. The mean ± SEM for at least 10^5 cells are shown. Data are representative of at least 5 replicate experiments.*

3.2.3 Death occurs in both infected and bystander DCs in vitro

The infection rate for BMDCs increased with the MOI, as measured by GFP^+ though not all cells were infected, even at very high MOIs (Fig.3-3A). The loss of viability in the infected DCs (defined by being GFP^+) increased with increasing MOI. In those wells that contained infected DCs, there was also a loss of viability in cells that were GFP^- (Fig.3-3B), defined as 'bystander' cells. The reduced viability of these uninfected DCs, which were in the same cultures with the infected cells, suggests that there may have been bystander death.

3.2.4 BMDC death can result from factors in the supernatant

The death of bystander cells suggested that there might have been toxicity associated with the culture media. Supernatants of infected cells were filtered and applied to uninfected BMDC cultures. The sterilised supernatants were able to cause death in these naïve DCs and the death ratio increases with the MOI of the previous infection where the supernatants were collected (Fig.3-4).

The pore size of the syringe filters was 0.4μm, which was smaller than the size of intact *Salmonella* (0.7-1.5μm in width and 2-5μm in length[207]). The filtration study reduced the possibility that the observed death in at least some bystanders was due to non-fluorescent bacteria. These filter-sterilised supernatants caused reduced viability of the naïve DCs, suggesting that at least some component of the bystander effect was due to factors contained in the infected culture supernatants.

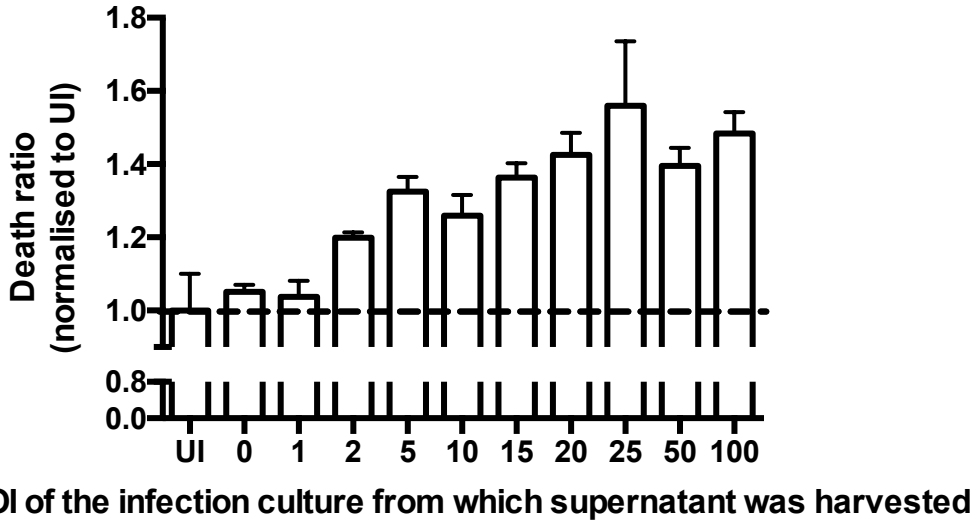

Figure 3-4: Cell death can be induced by sterilised supernatants.

Supernatants from infected cultures of increasing MOI (shown in Fig.3-3) were sterilised with 0.04µm syringe filters and were applied to naïve DCs and cells were then incubated for 2h. Viability was determined by flow cytometry using PI and CellTrace Violet staining. Death ratio was defined as described in Fig.3-3. One-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical analyses. No significance was observed in MOI 0-10, $p<0.05$ for MOI 15, 20 and 50, and $p<0.01$ for MOI 25 and 100. Each column represent 2 technical replicates. The mean ± SEM for at least 10^5 cells are shown. Data are representative of 2 experiments.

3.2.5 Inflammatory cytokines secreted by DCs are not responsible for the bystander death.

DCs are known to produce pro-inflammatory cytokines, such as TNF-α, IL-12, IL-1β[94, 208], and the supernatant of infected DCs is potentially rich in cytokines. TNF-α can cause apoptosis in cells[208, 209], and it was hypothesised that the cytokines produced by DCs mediated the death of bystander cells. To test this hypothesis, the level of pro-inflammatory cytokines in the supernatant of infected cells was quantified using the flow cytometry-based Cytokine Bead Array (CBA). The supernatant level of TNF-α, IL-6 and MCP-1 was increased after low MOI infections of DCs with *S.* Typhimurium*,* however, the concentration of the cytokines declined after the MOI exceeded a certain level which was likely due to cytotoxic effect of the high MOI (Fig.3-5A-C); the level of cytokines did not correlate with the increasing cell death seen with increasing MOI, in Fig.3-2B. These data suggested that cytokines produced by DCs are unlikely to be responsible for the cell death observed in uninfected 'bystander' DCs co-cultured with infected cells.

To further confirm if TNF-α is the key component in the supernatant to cause cell death, α-TNF-α antibody XT-22 was used to neutralise the TNF-α in the supernatant. After 2h infection with various MOIs of *S.* Typhimurium SL1344, DCs treated with α-TNF-α antibody did not show difference in death compared to untreated cells (Fig.3-5D). This result suggested that removing TNF-α from the supernatant does not save DCs from death.

To further examine whether cytokines contribute to the bystander death, DCs were treated with monensin to inhibit protein transport including cytokine secretion[210, 211] before infection. The supernatant was again harvested to confirm that cytokine release was successfully inhibited (Fig.S1). The level of bystander death was similar in monensin-treated and untreated groups, which suggests that the blocking of cytokine release does not rescue the bystander DCs from death, and that the key component in 'toxic' supernatant is unlikely to be any of the cytokines examined or indeed any monensin-inhibitable secreted proteins (Fig.3-5E).

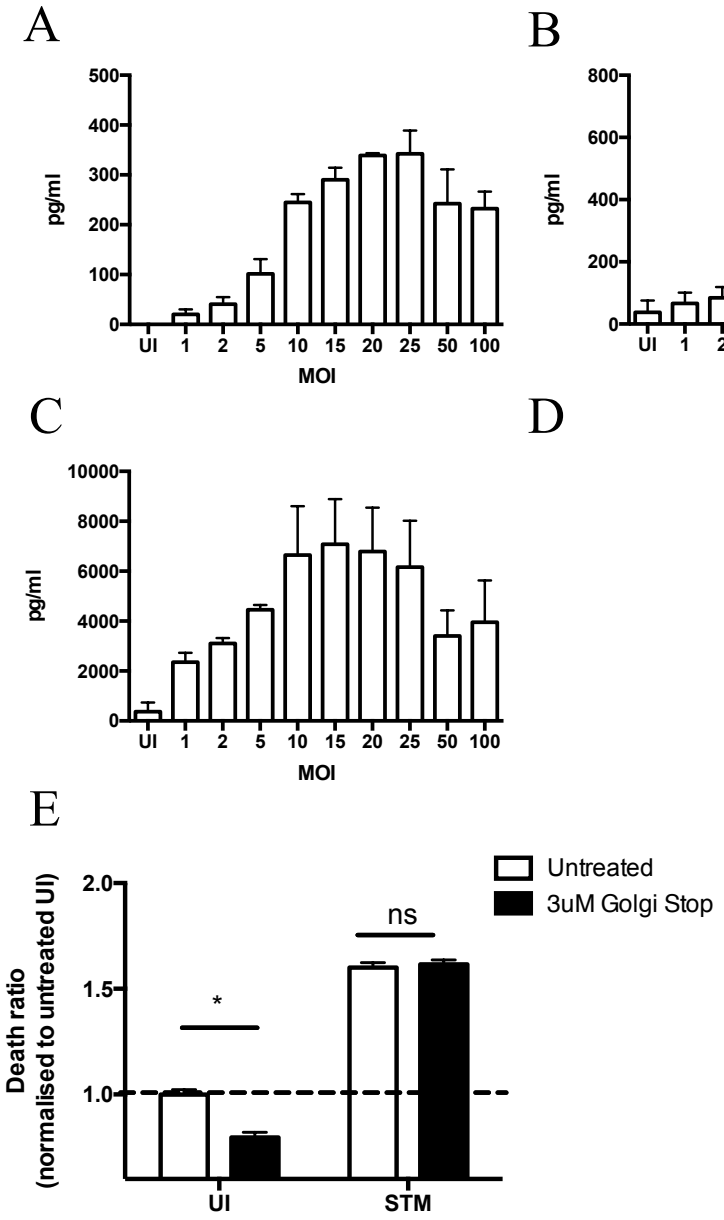

Figure 3-5: Blockade of cytokine release does not affect DC cell death.

Cytokines, including IL-6 (A), MCP-1 (B) and TNF-α (C) in the supernatants that were used in Fig.3-4 were determined by Cytometric Bead Array (CBA). α-TNF-α antibody (XT-22) were given to DCs 2h prior to the 2 hour infection of S. Typhimurium SL1344 at various MOIs to neutralise the TNF-α secreted into supernatants (D). Monensin was added to DCs at 3mM for 2h prior to the 2 hour infection (MOI 10) of S. Typhimurium SL1344 to stop protein transport, including cytokine secretion. Viability was determined by by flow cytometry using PI and CellTrace Violet staining (E). Each column represent 2 technical replicates. The mean ± SEM is shown. Two-way ANOVA with Tukey post-tests used for statistical analyses: *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001. *Data are representative of at least 2 experiments.*

3.2.6 LPS, SPI-1 and flagellin are involved in the DC death.

Since *Salmonella*-infected DCs appear to die independent of cytokine secretion, it is possible that death is linked directly to the infection process *per se*. To test whether live bacteria are required to cause death in DCs, DCs were either infected with SL1344 as previously, or co-incubated with a comparable dose of heat-killed SL1344. DCs co-incubated with HK SL1344 showed reduced cell death compared with DCs that were infected with live bacteria over the same period of time. This indicates that live bacteria contribute to death, but bacterial structural components may also have a cytotoxic effect, since DCs incubated with dead bacteria still demonstrate 1.5 fold increased death compared to uninfected controls.

One of the immunostimulatory bacterial components known to be present in *S.* Typhimurium is lipopolysaccharide (LPS). LPS, also known as endotoxin, is a major component of the cell wall of Gram-negative bacteria. To test the contribution of LPS to DC death, death of DCs from B6 mice were compared with those from $TLR4^{0/0}$ mice, which carried a single point mutation that rendered the TLR4 receptor non-responsive to LPS stimulation[212-214]. $TLR4^{0/0}$ DCs showed reduced viability after 2h infection with live SL1344 at MOI 20, and at levels that were similar to their wild-type counterparts, however $TLR4^{0/0}$ DCS were protected from death induced by heat-killed *S.* Typhimurium (Fig.3-6A). This result suggests that LPS/TLR4 mediated pathway is linked to *Salmonella*-induced death in DCs, however this pathway does not require the bacteria to be alive and other signalling pathway(s) are also involved and can cause death independent of that mediated through LPS.

To further investigate the killing of DCs by viable *S.* Typhimurium, *S.* Typhimurium strains that were deleted of SPI-1 (*invA),* SPI-II (*ssaR)* or flagellin (*fliCfljB)*, all of which were constructed in the SL1344 background, were analysed for their capacity to cause DC death. The gene *invA* encodes a key component of the SPI-1 Type Three Secretion System (T3SS) secretion apparatus[215, 216] and mutation or deletion in *invA* results in a reduction or loss *Salmonella* entry, termed invasion, into non-phagocytic cells[217, 218]. The Δ*invA* strain used in this project was tested in Madin-Darby canine kidney (MDCK) epithelial cell to confirm the invading was disabled (Fig.S2A). The gene *ssaR* encodes a key component of the SPI-2 T3SS, and is essential for the secretion of T3SS effectors and translocators from the SPI-2 secretion apparatus

under acidic pH[219]. SPI-2 is essential for the intracellular survival of *Salmonella* in 'professional' phagocytes. Intracellular growth of Δ*ssaR* mutant strain is deficient in macrophages[220], and the Δ*ssaR* strain used here was tested in bone marrow derived macrophages to confirm the mutation (Fig.S2B). FliC and FljB are two alternatively expressed flagellin proteins[176], composing core filament of the *Salmonella* flagella[221, 222], which provides mobility to the bacteria. The Δ*fliC*Δ*fljB* strain in this study was tested using 0.3% LB agar plates, which enables the visualization of motility. Compared with WT SL1344, the Δ*fliC*Δ*fljB* strain demonstrated loss of ability to spread over the plate surface, indicative of loss of mobility and consistent with the absence of the key flagellin proteins (Fig.S2C).

After 2 hours of infection, B6 BMDCs infected with Δ*invA* mutant or the Δ*fliC*Δ*fljB* mutant showed a decrease in the 'death ratio' compared with DCs infected with wild type *Salmonella* ($p<0.05$ or $p<0.001$ respectively), suggesting SPI-1 and flagellin are contributing to the early DC death. In contrast, the ratio of dead DCs in $TLR4^{0/0}$ BMDCs infected with Δ*invA* mutant and Δ*fliC*Δ*fljB* mutant was similar to that of the uninfected controls (Fig.3-6B), which suggests that SPI-1 and flagellin may act through a death pathway that involves signalling via TLR4, during an infection with live *Salmonella*.

3.2.7 Caspase-1-mediated pyroptosis probably contributes to DC death.

To further understand the host factors involved in dendritic cell death, BMDCs were generated from various mouse strains that are deficient in sensing bacteria or in death pathway intermediates. These include lines that are deficient for LPS sensing (TLR4, MyD88 and TRIF) (Fig-3.7A) or flagellin sensing (NLRC4, caspase-1/11 and IL-18) (Fig.3-7B) or death pathways, including apoptosis (caspase-8[123, 124]), pyroptosis (caspase-1/11)[99] or necroptosis (RIPK3 and MLKL)[140, 223] (Fig.3-7C). As RIPK3 causes embryo lethality in capase-$8^{-/-}$ mice[224], a mouse strain with a double knockout in RIK3 and caspase-8 was used, with $RIPK3^{-/-}$ alone used as a control strain for the double knockout.

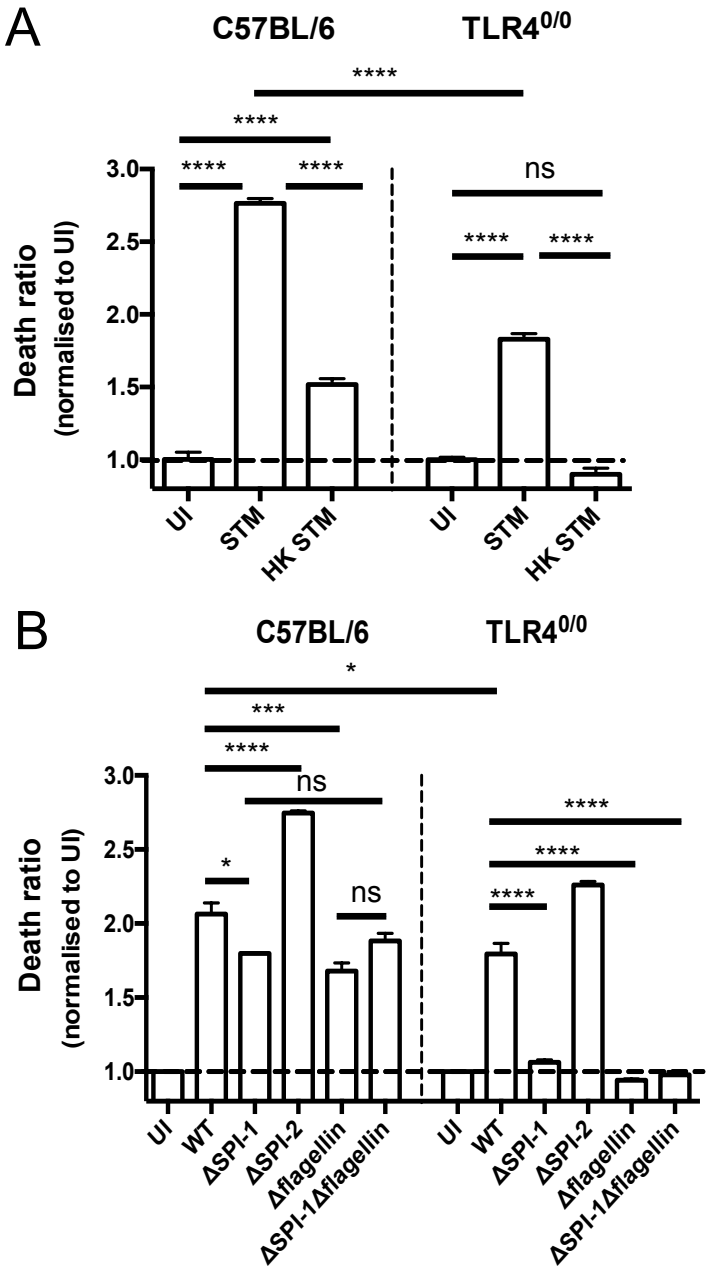

Figure 3-6: LPS, SPI-1 and flagellin are involved in the DC death.

BMDCs were derived from C57BL/6 (B6) and TLR4$^{0/0}$ mice. DCs were infected with WT STM or heat-killed (HK) STM (A) or STM deficient in SPI-1 (ΔinvA), SPI-2 (ΔssaR) or flagella (ΔfliCΔfljB) (B) at MOI 20. At 2h post infection cells are collected and analysed by flow cytometer. Viability was determined by flow cytometry using PI and CellTrace Violet staining. The mean ± SEM is shown. One-way ANOVA with Tukey post-tests was used for statistical analyses. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001. *Data are representative of at least 3 experiments.*

After 2 hours of infection of *S.* Typhimurium SL1344 at MOI 20, and consistent with previous experimental data (Fig.3-7A), $TLR4^{0/0}$ DCs showed reduced cell death compared with the death observed in B6 DCs ($p<0.0001$). MyD88 is situated in a signalling 'cascade' downstream from TLR4[167] and other TLRs[225, 226], however, unlike DCs from TLR4 mutant mice, DCs derived from $MyD88^{-/-}$ were not significantly protected from *Salmonella*-induced death. In contrast, DCs derived from mice that were mutant in the alternate downstream signalling molecule of TLR4, TRIF[168-170], were protected from killing by *S.* Typhimurium ($p<0.0001$) (Fig.3-7A). This data suggests that the DC death induced via the LPS/TLR4 pathway involves TRIF as the main downstream mediator, and not MyD88.

BMDCs from $ICE^{-/-}$ ($casp\text{-}1^{-/-}casp\text{-}11^{-/-}$) mice showed a very different pattern in PI and CTV staining after *S.* Typhimurium infection: the $CTV^{+}PI^{-}$ population decreased compared with that of C57BL/6 DCs, however, instead of having high number of cells that were PI^{+}, as seen with DCs from WT mice, many cells were located in $CTV^{+}PI^{int}$ (i.e. intermediate) area of the FACS plot (Fig.3-7B-D). This change of pattern suggests that caspase-1/11 may be involved in mediating *Salmonella*-induced death in the BMDCs.

It is accepted that NLRC4 activates caspase-1 after recognising intracellular flagellin[152], however, a deficiency in NLRC4 did not protect DCs from the *Salmonella*-induced death (Fig.3-7C-D). This suggests that the cleavage from pro-caspase-1 to caspase-1 by the NLRC4 inflammasome, which is activated by intracellular flagellin, is not a key component of the early DC death. $IL\text{-}18^{-/-}$ BMDCs were equivalent to B6 in DC death levels following *Salmonella* infection (Fig.3-7C-D), suggesting that the early DC cell death that is reduced in caspase-1/11 knockout mice, may not be mediated through IL-18, a key effector activated by functional caspase-1[99]. However, disabling the pyroptosis death pathway by depleting caspase-1/11 does not completely rescue the DCs from *Salmonella*-mediated death, which suggests other death pathways may also be involved.

To further explore the reduced cell death observed in DCs from $ICE^{-/-}$ and to examine other death pathways, BMDCs were cultured from caspase-$11^{-/-}$, $MLKL^{-/-}$, $RIPK3^{-/-}$ and $RIPK3^{-/-}CASP8^{-/-}$ mice and these DCs were studied in the *Salmonella* infection

assay. Compared to C57BL6 BMDCs, caspase-11$^{-/-}$ BMDCs showed a similar level of reduction in cell death after infection as seen in ICE$^{-/-}$, which suggests the involvement of caspase-11 in this early cell death. In contrast, DCs from RIPK3$^{-/-}$, RIPK3$^{-/-}$CASP8$^{-/-}$ and MLKL$^{-/-}$ showed no difference to those of B6 DCs in viability measured 2h after infection, suggesting that apoptosis mediated via caspase-8, and necroptosis mediated via RIPK3 and MLKL, may not be participating in this early cell death of DCs.

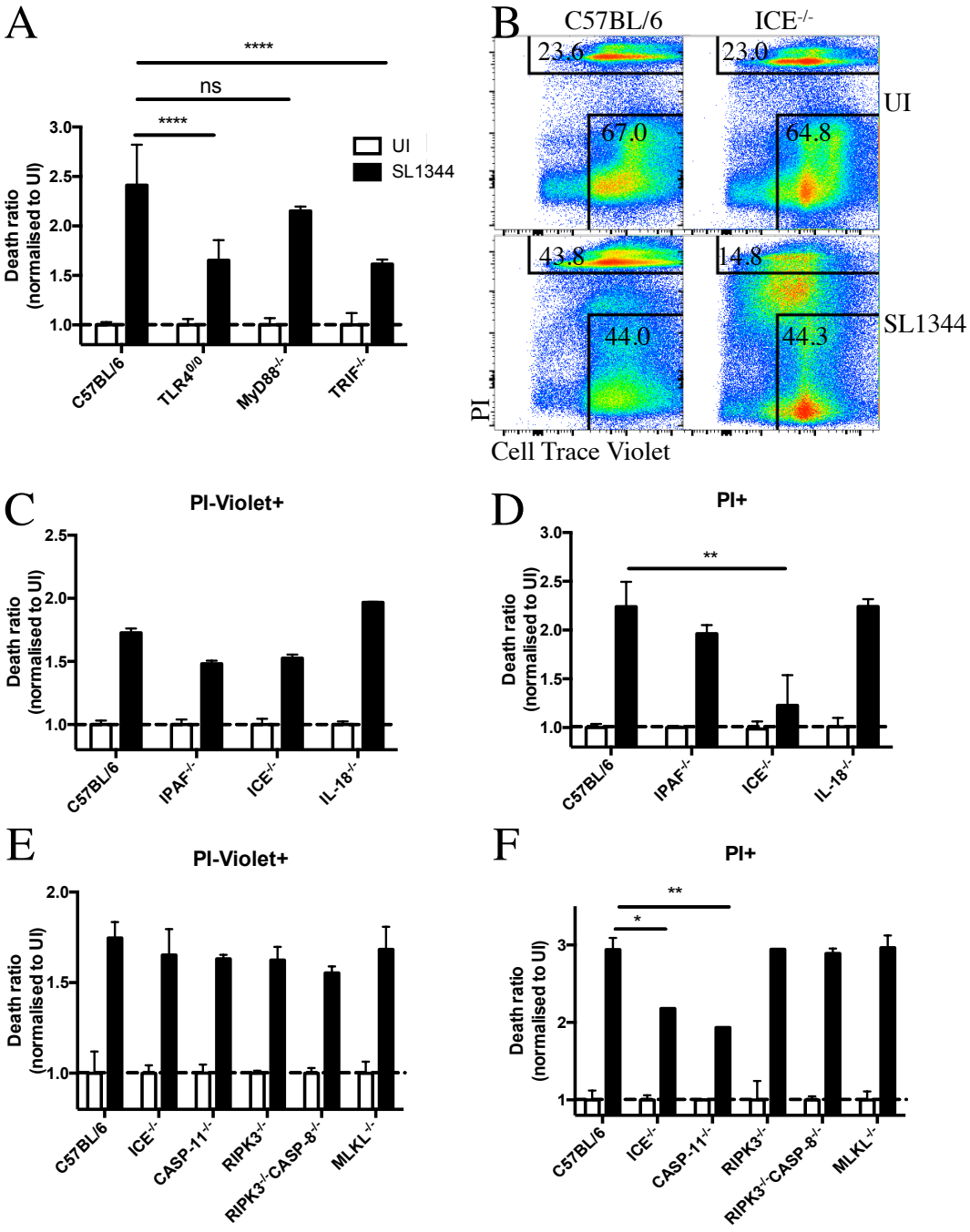

Figure 3-7: Caspase-1 and caspase-11 may be involved in the early DC cell death.

BMDCs were derived from C57BL/6 (B6), TLR4$^{0/0}$, MyD88-/-, TRIF-/- (A), IPAF$^{-/-}$ (NLRC4$^{-/-}$), ICE$^{-/-}$ (CASP-1$^{-/-}$-CASP-11$^{-/-}$), IL-18$^{-/-}$ (B,C,D), CASP-11$^{-/-}$, RIPK3$^{-/-}$, RIPK3$^{-/-}$ CASP8$^{-/-}$ and MLKL$^{-/-}$ mice (E,F). DCs were infected with STM SL1344 at MOI 20. At 2h post infection cells are collected and analysed by flow cytometer. Viability was determined by flow cytometry using PI and CellTrace Violet staining. Death ratio were calculated on two populations shown in Fig.3-2B: PI-Cell Trace Violet^{+} as the previous figures (A,C,E) and PI^{+} (D,F) . The mean ± SEM is shown. Two-way ANOVA with Tukey post-tests was used for statistical analyses. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001. *Data are representative of at least 2 experiments.*

3.3 Discussion

DCs play a central role in immunity against pathogens, linking innate and adaptive immunity[82, 94, 208]. In both human and mice, T cell-mediated immunity is important in host protection against *Salmonella*[8, 73, 227], and emphasizes the important role DCs may play in controlling *Salmonella* as the inducer of the adaptive immune system[87-95]. It is hypothesised that the inability of some strains of mice, such as BALB/C and C57BL/6 to control *Salmonella* infections could reflect the failure of DCs to drive adaptive immune protection. *In vitro* studies have addressed the importance of DCs in *Salmonella* infection[82, 94, 208]. However, depletion of DCs in mice induces the expansion of other immune cells, such as monocytes and neutrophils, which participate in controlling the infection[228, 229]. It is therefore difficult to ascertain whether effects of DC depletion relate to direct roles for these cells during *Salmonella* infection or indirect effects as a consequence of regulating other cell types.

In this study, *Salmonella* infection was associated with an apparent reduction in different splenic DC subsets. The reduction in DC numbers might be explained in several ways. DCs are able to circulate, where circulation and residency are controlled through a variety of receptors expressed on the surface of the cells, which enable recruitment to lymphoid and peripheral tissues by various chemokines[230]. As *S.* Typhimurium causes a systemic infection in mice, the DCs may have migrated to other tissues that were also infected. The apparent reduction in DC numbers could be explained by a loss of cell surface markers that facilitate identification. LPS is known to induce a down-regulation of CD11c in BMDCs and *ex vivo* DCs via MyD88[231, 232]. It is possible that infection by *Salmonella* triggered the down-regulation of CD11c, which was used as a marker to define DC subsets, resulting in more cells defined as $CD11c^-$. A third explanation is that *Salmonella* can induce death in DCs[114, 115].

To study the impact of *Salmonella* infection on DCs, an *in vitro* bone marrow derived dendritic cells (BMDC) system was used. Hematopoietic growth factors, usually Granulocyte macrophage colony stimulating factor (GM-CSF)[196-199] or *fms*-like tyrosine kinase 3 ligand (Flt3L)[194, 195, 233, 234], were used to stimulate bone marrow cells to differentiate into different types of dendritic cells.[235]. Phenotypically, GM-CSF derived BMDCs resembled monocyte-derived DCs *in vivo*, whereas Flt3L derived BMDCs shared biomarkers with cDCs and pDCs[195, 236].

By using the *in vitro* BMDC system, the research presented here shows that *Salmonella* infection at moderate MOIs (5-25) can cause death in DCs, revealed through CTV and PI staining, even though DCs are considered to be a 'permissive site' for *Salmonella* replication[102, 103]. This degree of rapid cell death increased as the MOI increased, however, populations DCs remained alive, even in infected cultures exposed to very high MOI (100), implying that some of the DCs are protected from either infection and/or from death. It is reported that the expression level of Bcl-A1, which is an anti-apoptotic protein, is up-regulated in infected DCs (Fu Guo, University of Melbourne PhD Thesis). The activation of anti-apoptotic pathways may help explain why some cells are resistant to even high MOIs.

Interestingly, it was found that, in *in vitro* assays, *S.* Typhimurium do not only cause death in the DCs that they have infected or actively invaded, but also in bystander cells that have not been directly infected. Where live bacteria were removed from the supernatants of infected cultures by filtration, supernatants replicated the death-inducing effect in naïve BMDCs, which suggested that the bystander death caused by *S.* Typhimurium infection is due to a component in the supernatant. However, this supernatant from infected culture is a complicated environment, which contains not only what is released by cells but also bacteria components, e.g. LPS or flagellin. It cannot be concluded from the supernatant treatment what in the supernatant is able to cause bystander death in DCs.

It is hypothesised that cytokines, such as TNF-α, were released once the DCs were stimulated with *Salmonella*, which further induced apoptosis. However, the low cytokine levels in high MOI (50-100), which is possibly due to the loss of cytokine producing cells, could not explain the high death ratio in the bystanders in cells infected with MOI 100. Also, pre-treatment of infected DCs with monensin that inhibits protein transport[210, 211] or the use of anti-TNF-α neutralising antibodies, did not rescue DCs from being killed by *S.* Typhimurium, suggesting that this bystander death is likely not caused by cytokines released by DCs as part of host defence.

Incubating DCs with heat-killed S. Typhimurium (HKST) lead to a reduced level of death in DCs, compared with the level seen in live *S.* Typhimurium infections,

suggesting that there may have been two types of cell death. One contributed by bacteria structural components in HKST, and the other present in live bacteria and driven by the invasion process. Infection of BMDCs derived from $TLR4^{0/0}$ mice, which are unable to respond to LPS[212-214], suggest that the death caused by structural components might be attributable to LPS. Though LPS triggers the release of TNF-α or IL-1β, which induce apoptosis or necroptosis[237-239], the monensin experiments suggest that blocking the release of the cytokines did not reduce the proportion of killed bystander cells, after cells were infected with *S.* Typhimurium. The lack of effect of monensin treatment suggests that TLR4 may cause death through other mechanisms independent of cytokines[239].

TLR4, after sensing LPS, recruits MyD88 and activates the canonical MyD88 signaling pathway[167] and induces the production of cytokines including TNF-α, IL-6, IL-12p40 and IFN-γ. TLR4 is also able to signal through TIR-domain-containing adapter-inducing interferon-β (TRIF) in addition to MyD88, which regulates the secretion of Type I IFNs, IL-10, NF-kB and IFN-γ[159, 166]. DCs generated from mouse strains that are deficient for downstream effectors of TLR4, MyD88 or TRIF were used in the *in vitro* killing assay, and $TRIF^{-/-}$ cells responded the same as DCs from $TLR4^{0/0}$ mice to *Salmonella* infection, while $MyD88^{-/-}$ DCs did not show similar protection from death mediated by *Salmonella* infection. This result suggests the death induced by LPS/TLR4 is mostly mediated via the TRIF pathway and not via MyD88.

The *Salmonella* spp. T3SSs form a needle-like complex that senses host cell and delivers effector proteins into the cell. There are two T3SSs, encoded by SPI-1 and SPI-2, which are expressed at different stages to support invasion or intracellular survival of bacteria, respectively[38, 133, 134, 139]. Mutant strains that did not express the SPI-1 T3SS apparatus component InvA, without which the bacteria is not able to translocate effectors[240], showed an impaired ability to cause death in DCs compared to wild type *S.* Typhimurium. This impairment in causing death suggests that SPI-1 is involved in the active killing that requires viable bacteria. Whereas a mutant *S.* Typhimurium strain that did not express SPI-2 T3SS apparatus component SsaR, without which the bacteria is not able to survive in the cells[219], showed an increased ability to cause DC death compared with WT ($p<0.001$). This increased ability of

killing suggested that SPI-2 might be able to trigger anti-death response, when it helps *Salmonella* survive within the DCs.

Flagella are complex surface bacterial organelles that provide motility to *Salmonella* and the lack of flagellin, a key component of flagella[221, 222], leads to a reduced ability to cause death in DCs. A mutant *S.* Typhimurium strain that did not express InvA and flagellin showed no further reduction in DC death compared to the Δ*invA* mutant or Δ*fliC*Δ*fljB* mutant, implying that SPI-1 and flagellin may be involved in the same pathway that live bacteria utilize to kill host cells. It is possible that active killing by the bacteria mainly relies on the flagellin secreted by SPI-1 encoded T3SS[133, 134], but that the flagellin entering into the cytosol independent of SPI-1 is also playing a small role. In addition, the data suggest that the sensing of LPS through TLR4 may facilitate this independent entry in an unknown way. In summary, LPS, SPI-1 and flagellin are required for maximal DC death induced by *Salmonella* infection.

NLRC4 forms part of a complex called inflammasome. It senses cytosolic flagellin and activates the proteolytic enzyme caspase-1, also called interleukin (IL)-1β-converting enzyme (ICE)[146]. Caspase-1 associates with NLRC-4 via CARD-CARD interactions, and adaptor protein ASC is required as part of the inflammasome[132-134]. Activated caspase-1 converts the pro-inflammatory cytokine IL-18 into the mature form[147]. Also, as mentioned previously, the active form of caspase-1 can drive pyroptosis in *Salmonella*-infected cells[99, 153]. However, in data shown here, NLRC4 was not required for the early DC death caused by *Salmonella*, which suggests that the activation of caspase-1 is not integral in this early DC death. It was observed that there was a reduction in death in infected DCs generated from ICE$^{-/-}$ (caspase-1$^{-/-}$ caspase-11$^{-/-}$) mice, but not from IL-18$^{-/-}$ mice, which suggests the death may be mediated by either caspase-1 or caspase-11. Further experiments with caspase-11$^{-/-}$ BMDCs suggested that caspase-11 is mediating the early DC death. Given that NLRC4 activating caspase-1 seemed to be unnecessary, it is likely that the early death is mainly mediated via caspase-11. However, it cannot be concluded whether caspase-1 is involved. Further experiments with a specific caspase-1 single knockout will be needed to draw this conclusion. It is reported that intracellular LPS activates caspase-11 non-canonically[173, 241-246], however some studies claimed that TLR4 and TRIF were crucial for the activation of caspase-11[173, 241, 242] whereas others reported that

this non-canonical activation is TLR4 independent[243-246]. During pyroptosis, spherical protrusions are formed, which are also observed in apoptosis. However, instead of forming apoptotic bodies and being phagocytised, cells that undergo pyroptosis continue to swell and eventually rupture and release cellular contents into the media[126]. It was reported that ASC released by this process can be taken up by macrophages and activate caspase-1 within the phagocytosing cell[247]. The observed bystander on DCs could have resulted from ASC released into the environment from those DCs that were killed via pyroptosis. As ASC is also part of caspase-11 inflammasome, it is likely that this mechanism applies to caspase-11 mediated pyroptosis as well.

BMDCs derived from RIPK3$^{-/-}$CASP8$^{-/-}$. RIPK3$^{-/-}$ and MLKL$^{-/-}$ did not show a clear reduction in WT *S.* Typhimurium-induced cell death, suggesting that apoptosis and necroptosis may not be participating in the early DC death phenomenon observed in this study.

In summary, *S.* Typhimurium is able to cause programmed cell death, which is most likely to be pyroptosis, in DCs within a short period after infection, mostly via SPI-1 and flagellin. However, the bacteria are also preventing some infected cells from death via SPI-2, probably such that they can further replicate inside of the cells. This brings a focus onto the question as to whether the host, or the *Salmonella* bacterium, most benefits from DC death? It could be hypothesised that *Salmonella* eliminate the majority of DCs to prevent DCs from full function, but sufficient cells remain protected from death to ensure a *Salmonella* replicative niche.

Chapter 4

The role of IFN-γ pathways in *Salmonella* immunity

4.1 Introduction

Understanding the mechanism of host immune protection against *Salmonella* infection is important for the better management of diseases caused by pathogenic *Salmonella*. IFN-γ is critical for the early control of *Salmonella* infection[71, 141, 143-145]. Amongst other signalling pathways, previous studies have shown that intracellular flagellin leads to potent activation of IFN-γ production. Upon infection with *Salmonella*, NLRC4 inflammasomes sense intracellular flagellin and cleave pro-caspase-1 into its active form, leading to the release of pro-inflammatory cytokine IL-18[129]. The released IL-18 from infected cells, such as dendritic cells and macrophages, induces IFN-γ secretion in subsets of T cells and NK cells[152]. Previous work[152] shows that $CD8^+$ T cells from naïve C57BL/6 mice release IFN-γ within 2 hours of intravenous injection with heat-killed *S.* Typhimurium (HKST) SL1344. The responding $CD8^+$ T cells include non-cognate memory $CD8^+$ T cells, which are $CD44^+CXCR3^+CD62L^+$. Interestingly, antigen specificity to *Salmonella* is not required, as a similar response can be induced in $CD8^+$ T cells from gBT-I transgenic mice, which are genetically engineered to express a single T cell receptor (TCR) that specifically recognises the immunodominant epitope of herpes simplex virus type 1 glycoprotein B, further confirming that this rapid IFN-γ release occurs independently of TCR recognition of *Salmonella* antigens in the responding $CD8^+$ T cells.

In contrast to wild type *S.* Typhimurium that causes an acute, lethal infection in C57BL/6 mice, infection with attenuated *S.* Typhimurium strain BRD509 (an auxotroph for aromatic compounds[69]) leads to a chronic infection that can be controlled and eventually cleared within 10-12 weeks in infected mice[71]. This extended infection provides a time frame to study how different immune components contribute to effective immunity against *Salmonella* infection[71]. Adoptive transfer of *in vivo* HKST-activated gBT-1 memory cells prolongs survival and provides partial but non-lasting protection against *S.* Typhimurium BRD509 infection in IFN-$\gamma^{-/-}$ mice[152], suggesting a functional role for this rapid IFN-γ response in host immunity against *Salmonella*. Thus, it was hypothesized that the NLRC4 inflammasome-dependent IFN-γ induction pathway may be important in conferring host immunity against *Salmonella* infection. In the studies reported in this chapter, this hypothesis will be tested using $ICE^{-/-}$ (caspase-$1^{-/-}$caspase-$11^{-/-}$) and IL-$18^{-/-}$ mice that are deficient for NLRC4 inflammasome-dependent IFN-γ[152].

To place NLRC4-dependent IFN-γ induction in context, the contribution of other signalling pathways to total IFN-γ response will also be investigated. In particular, the TLR4 pathway senses LPS that is present on the outer membrane of Gram-negative bacteria, and leads to the activation of NF-κB via either the MyD88 or TRIF pathways. The activation of the TLR4/LPS pathway regulates pro-inflammatory and anti-inflammatory cytokines, including IFN-γ. Thus, $TLR4^{0/0}$ mice, which carried a single point mutation that rendered the TLR4 receptor non-responsive to LPS stimulation, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice will be used to dissect TLR4-mediated IFN-γ responses during *Salmonella* infection.

Interestingly, purified flagellin can recapitulate NLRC4-dependent IFN-γ responses in non-cognate memory $CD8^+$ T cells and NK cells induced by HKST[152], indicating that the detection of flagellin plays an important role in activating host immunity against *Salmonella*. However, it is reported that *Salmonella* represses the expression of flagellin after phagocytosis by macrophages or dendritic cells to prevent further immune responses[40]. *S.* Typhimurium exhibits flagellar phase variation by alternatively expressing two flagellin proteins, encoded by *fliC* and *fljB*. The FliC and FljB proteins share substantial sequence homology (75%-85%)[180] but are antigenically distinct[177]. The implication of this phase variation on flagellin-mediated NLRC4 inflammasome activation is unclear and will be investigated using *S.* Typhimurium mutant strains that are deficient in FliC or FljB individually.

Overall, the aims in this chapter are:

a. to understand if the flagellar phase variant influences the secretion of IFN-γ from T cells and NK cells;

b. to study the contribution of NLRC4 pathway to host immunity in *Salmonella* infection;

c. to study the contribution of the TLR4 pathway to host immunity in *Salmonella* infection.

4.2 Results

4.2.1 FliC and FljB both contribute to the induction of IFN-γ secretion

To understand if flagellar phase variation affects the rapid release of IFN-γ by $CD8^+$ T cells after the *Salmonella* infection, mice were treated with heat-killed wild-type *Salmonella* SL1344, or heat-killed *Salmonella* that were deficient in either or both of FliC and FljB. Heat-killed *Salmonella* with a flagellar complementing plasmid pls408 (PLS408) encoding *fliC* was used as a positive control. Two hours after the injection of heat-killed *Salmonella*, $CD44^{high}$ splenic $CD8^+$ T cells secreted IFN-γ in response to wild type, PLS408, Δ*fljB*, Δ*fliC*, but not PBS and Δ*fliC*Δ*fljB* (Fig.4-1). As expected and as reported before, this result shows that flagellin is responsible for triggering rapid IFN-γ secretion in non-cognate memory $CD8^+$ T cells, as a strain that is deficient in flagellin completely (Δ*fliC*Δ*fljB*) is unable to trigger this reaction[152]. The mutants expressing either FliC or FljB were still able to stimulate the IFN-γ secretion, but not to the same level as WT *Salmonella* or the complemented strain (Fig.4-1), suggesting that FliC and FljB are both contributing to the IFN-γ burst in a non-redundant manner.

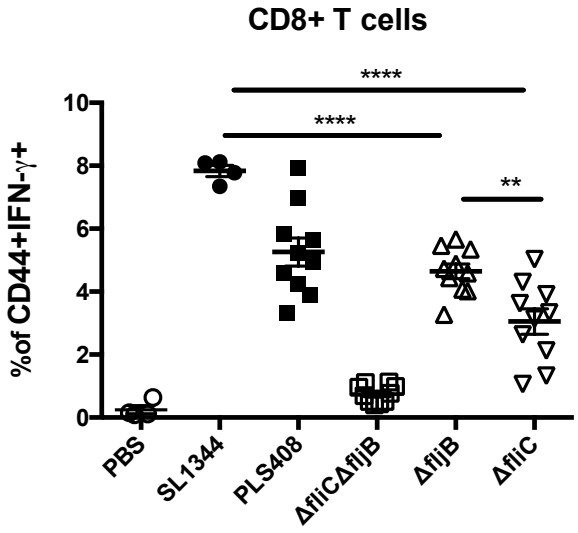

Figure 4-1: Both FliC and FljB contribute to triggering the secretion of IFN-γ.

*Mice were i.v. infected with PBS or 10^8 cfu of HK STM SL1344, HK STM deficient in FliC and FljB with a flagellar complement plasmid pls408 (PLS408), HK STM deficient in FliC and FljB (ΔfliCΔfljB), HK STM deficient in FliC (ΔfljB) or HK STM deficient in FljB (ΔfliC). 2 hours later, splenocytes were harvested and IFN-γ secretion by CD8+ T cells was assessed by FACS. Symbols represent data from individual animals, pooled from 3 independent experiments. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM). One-way ANOVA followed by Tukey's test were used for statistical analyses. **$p<0.01$ ****$p<0.0001$.*

4.2.2 Caspase-1 and IL-18 have a minor role in the control of *Salmonella* infection

To study the contribution of molecules downstream of NLRC4 in host immunity against *Salmonella* infection, C57BL/6 (B6), ICE$^{-/-}$ (caspase-1$^{-/-}$caspase-11$^{-/-}$) and IL-18$^{-/-}$ mice were infected with *S.* Typhimurium BRD509 and the mice killed, and organs removed, at different time points after infection. As described previously, B6 mice are capable of clearing the bacteria within 10-12 weeks and at the dosage used (200 cfu), mice did not experience weight loss as a consequence of the infection (Fig.4-2A). Instead, splenomegaly was observed in B6 mice around 2-5 weeks after infection, which was at least in part due to an influx or proliferation of immune cells, including neutrophils and macrophages, as well as B cells and T cells[248] (Fig.4-2B). The bacteria load increased in the first two weeks and plateaued, after that *Salmonella* was gradually cleared within 12 weeks (Fig.4-2C-D). Surprisingly, although the bacterial load in the spleen and liver of ICE$^{-/-}$ and IL-18$^{-/-}$ mice were 10-fold higher than that in WT mice at 3 weeks after infection, ICE$^{-/-}$ and IL-18$^{-/-}$ mice were also able to control and clear the bacteria by 12 weeks post-infection (Fig.4-2C-D). These two strains of mice (ICE$^{-/-}$ and IL-18$^{-/-}$) did not suffer from weight loss (Fig.4-2A) and the extents of splenomegaly were similar to WT mice (Fig.4-2B). This result suggests that the caspase-1/IL-18 pathway plays a minor and non-essential role in controlling *Salmonella* BRD509 infection.

4.2.3 Altered pro-inflammatory cytokines in *Salmonella*-infected ICE$^{-/-}$ and IL-18$^{-/-}$ mice.

To further study the contribution of the caspase-1/IL-18 pathway to regulating inflammatory cytokine secretion during *Salmonella* infection, blood samples were collected from the same experiment as described in Fig.4-2 and were used to determine the serum cytokine levels by cytokine bead array (CBA). In wild type C57BL/6 mice, the serum IFN-γ level increased and peaked at 2 weeks post-infection (Fig.4-3A), a time frame that coincided with peak of the bacterial load in the spleen and liver (Fig.4-2C-D). In contrast, the serum level of IFN-γ detected in ICE$^{-/-}$ and IL-18$^{-/-}$ mice was reduced compared to wild-type mice at week 2, but both followed the

same pattern as WT mice and peaked at week 3 and week 2 post-infection, respectively (Fig.4-3A). The peak level of IFN-γ in ICE$^{-/-}$ mice coincided with the peak bacterial load in in the spleen and liver (Fig.4-2C-D), which was one week later than observed for WT B6 mice and IL-18$^{-/-}$. This delay in peak IFN-γ might reflect that caspase-1 is not only involved in this inflammasome pathway, but also in pyroptosis, and that the pyroptosis initiated via caspase-1 contributed to the bacteria control. This result suggests that deficiency in the caspase-1/IL-18 pathway leads to reduced systemic level of IFN-γ in *Salmonella*-infected mice, however the remaining amount of IFN-γ was sufficient for the host to control bacteria.

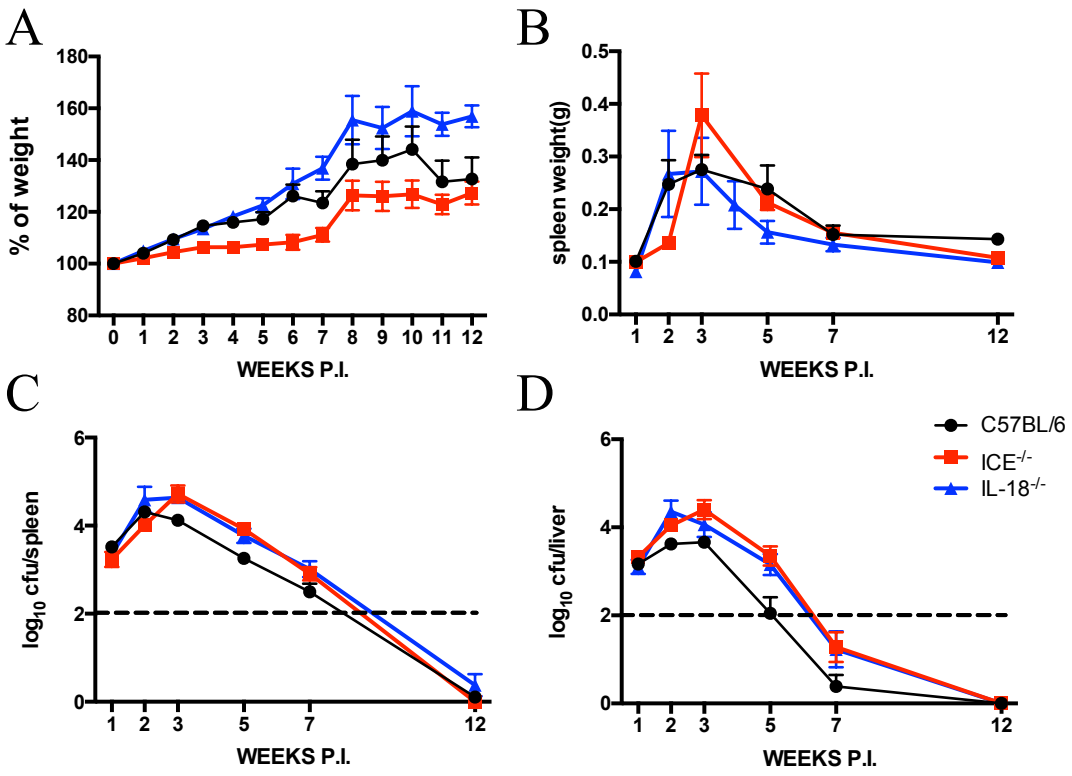

Figure 4-2: Caspase-1 and IL-18 have minor roles in Salmonella control.

C57BL/6 (black solid dots), ICE$^{-/-}$(caspase-1$^{-/-}$caspase-11$^{-/-}$) (red solid squares) and IL-18$^{-/-}$ (blue solid upper triangles) mice were infected with 200cfu BRD509 intravenously. Mice were weighed every week (A) and organs were taken on week1, 2, 3, 5, 7 and 12. Spleens were weighed to determine the splenomegaly (B). Spleens and livers were homogenised to determine the bacteria counts (C-D) and blood were taken for further experiments. 12-15 mice were used for each strain at each time point and data was pooled from 3 independent experiments. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM).

The systemic level of other inflammatory cytokines followed similar patterns to IFN-γ but were of different magnitudes. Whereas the serum level of TNF-α in IL-18$^{-/-}$ mice was similar to wild-type mice after *Salmonella* infection, in ICE$^{-/-}$ mice, serum TNF-α was reduced compared to both WT and IL-18$^{-/-}$ mice (Fig.4-3C). This result suggests that caspase-1 and IL-18 may regulate TNF-α production via different pathways. On the other hand, the serum level of monocyte chemotactic protein-1 (MCP-1) in ICE$^{-/-}$ and IL-18$^{-/-}$ mice was not different from WT mice, suggesting that MCP-1 production is most likely independent of caspase-1/IL-18 pathway (Fig.4-3B).

4.2.4 IFN-γ is sufficient for host immunity against *Salmonella* independent of IL-18.

Results to-date suggest that IL-18$^{-/-}$ mice concurrently exhibit reduced IFN-γ responses and reduced control of *Salmonella* within the early phase of infection, but are ultimately able to clear bacteria within a time frame comparable to their WT B6 counterparts. One possibility is that the residual amount of IFN-γ produced in IL-18$^{-/-}$ mice, albeit significantly reduced, can confer sufficient control of bacterial growth *in vivo*. To test this hypothesis, anti-IFN-γ antibody (HB-170-15) was used to neutralise IFN-γ in *Salmonella*-infected IL-18$^{-/-}$ mice. Compared to PBS-treated control mice, treatment with IFN-γ antibody induced significantly increased weight loss (Fig.4-4A), splenomegaly (Fig.4-4B) and higher bacterial load in the spleen (Fig.4-4C) and liver in both IL-18$^{-/-}$ and B6 mice (Fig.4-4D). The absence of IFN-γ in the serum confirmed the effective depletion of IFN-γ by antibody treatment (Fig.4-4E). This result suggests that the depletion of residual IFN-γ in IL-18$^{-/-}$ mice abolished host immunity against *Salmonella* infection.

4.2.5 TLR4 pathway contributes to the early control of *Salmonella* infection.

To better understand the NLRC4-independent source of IFN-γ, the contribution of the TLR4 pathway to host immunity against *Salmonella* infection was studied. C57BL/6, IFN-γ$^{-/-}$, TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were infected and examined on week 3 and week 12 post-infection. As expected, IFN-γ$^{-/-}$ mice became severely sick and succumbed to infection within 4 weeks, but none of the other strains showed disease symptoms including weight loss (Fig.4-5A). All mice developed splenomegaly at weeks 3 post-infection, albeit to different extent, and all recovered at

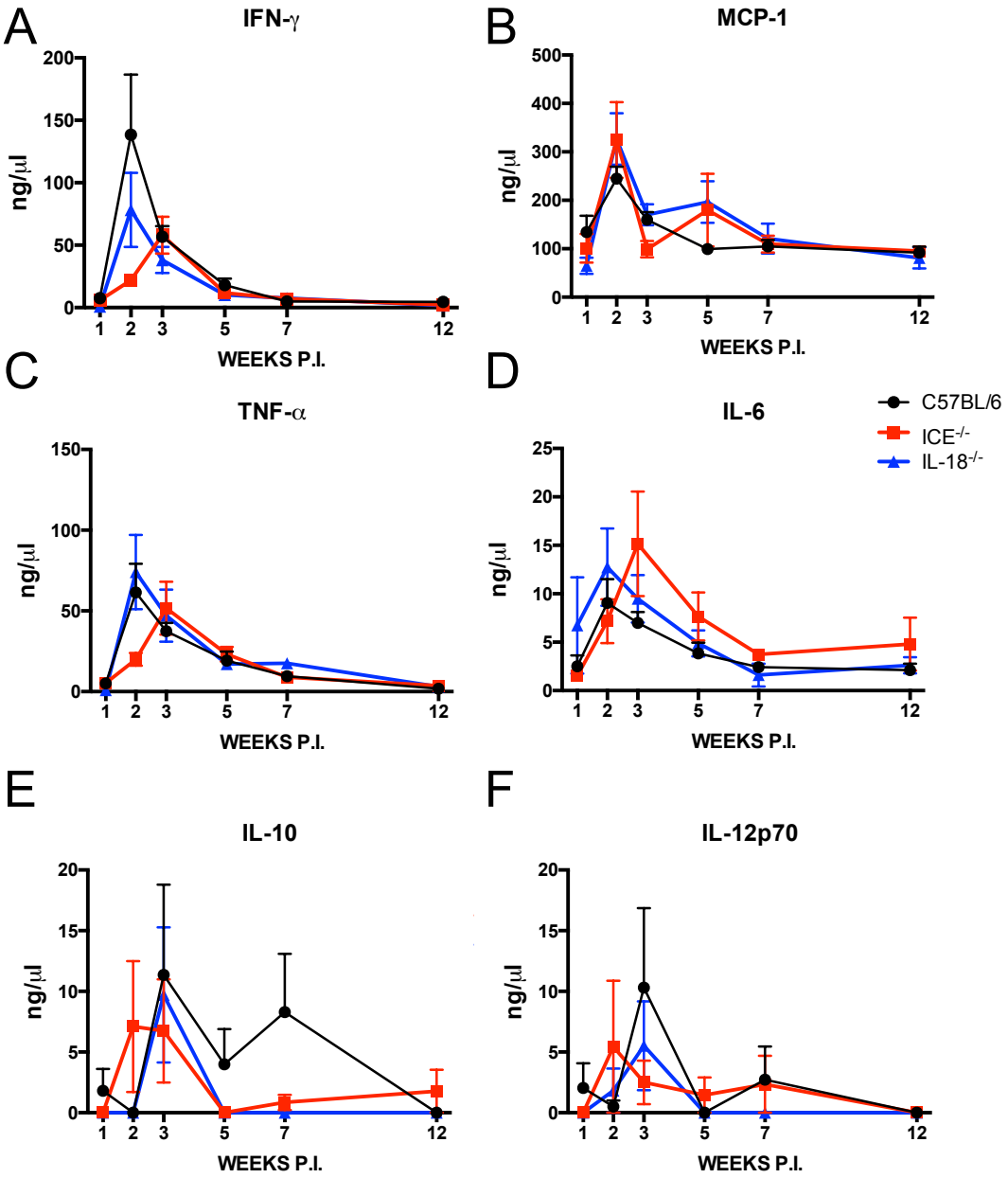

Figure 4-3: Impaired levels of pro-inflammatory cytokines were detected in ICE$^{-/-}$ and IL-18$^{-/-}$ mice.

C57BL/6 (solid dots), ICE$^{-/-}$(caspase-1$^{-/-}$caspase-11$^{-/-}$) (solid squares) and IL-18$^{-/-}$ (solid upper triangles) mice were infected with 200cfu BRD509 intravenously (Fig.4-2). Blood was taken at various time points to determine the pro-inflammatory cytokine levels in serum by cytokine bead array (CBA). IFN-γ (A), MCP-1 (B), TNF-α (C), IL-6 (D), IL-10 (E) and IL-12p70 (F) were tested in the assay. 12-15 mice were used for each strain at each time point and data was pooled from 3 independent experiments. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM).

week 12 (Fig.4-5B).

Three weeks after infection, IFN-$\gamma^{-/-}$, TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice showed very high bacterial loads in both the spleens and liver (Fig.4-5C-D). It is known that IFN-γ is essential for controlling *Salmonella* infection and TLR4 plays an important role in recognising LPS of *Salmonella* to trigger innate immune responses. However, TLR4$^{0/0}$ mice were able to survive and clear a bacterial load that was almost as high as that observed in IFN-$\gamma^{-/-}$ mice, suggesting that some mechanism independent of TLR4 pathway contributed to the control of the bacterial load and was beneficial to survival. MyD88 and TRIF mediate two separate pathways downstream of TLR4, yet TRIF$^{-/-}$ showed approximately 10-fold higher bacterial load in the spleen and liver compared to that of MyD88$^{-/-}$ mice, suggesting that in *Salmonella* infection, the TRIF pathway contributes more to the control of bacteria than the MyD88 pathway. However, the systemic level of IFN-γ was not reduced but instead increased in TLR4$^{0/0}$, MyD88$^{-/-}$ or TRIF$^{-/-}$ mice compared to wild-type mice (Fig.4-5E), indicating that although the TLR4 pathway play a role in the control of *Salmonella* infection, it is likely that this contribution to control of the bacteria is mediated through mechanisms independent of IFN-γ production.

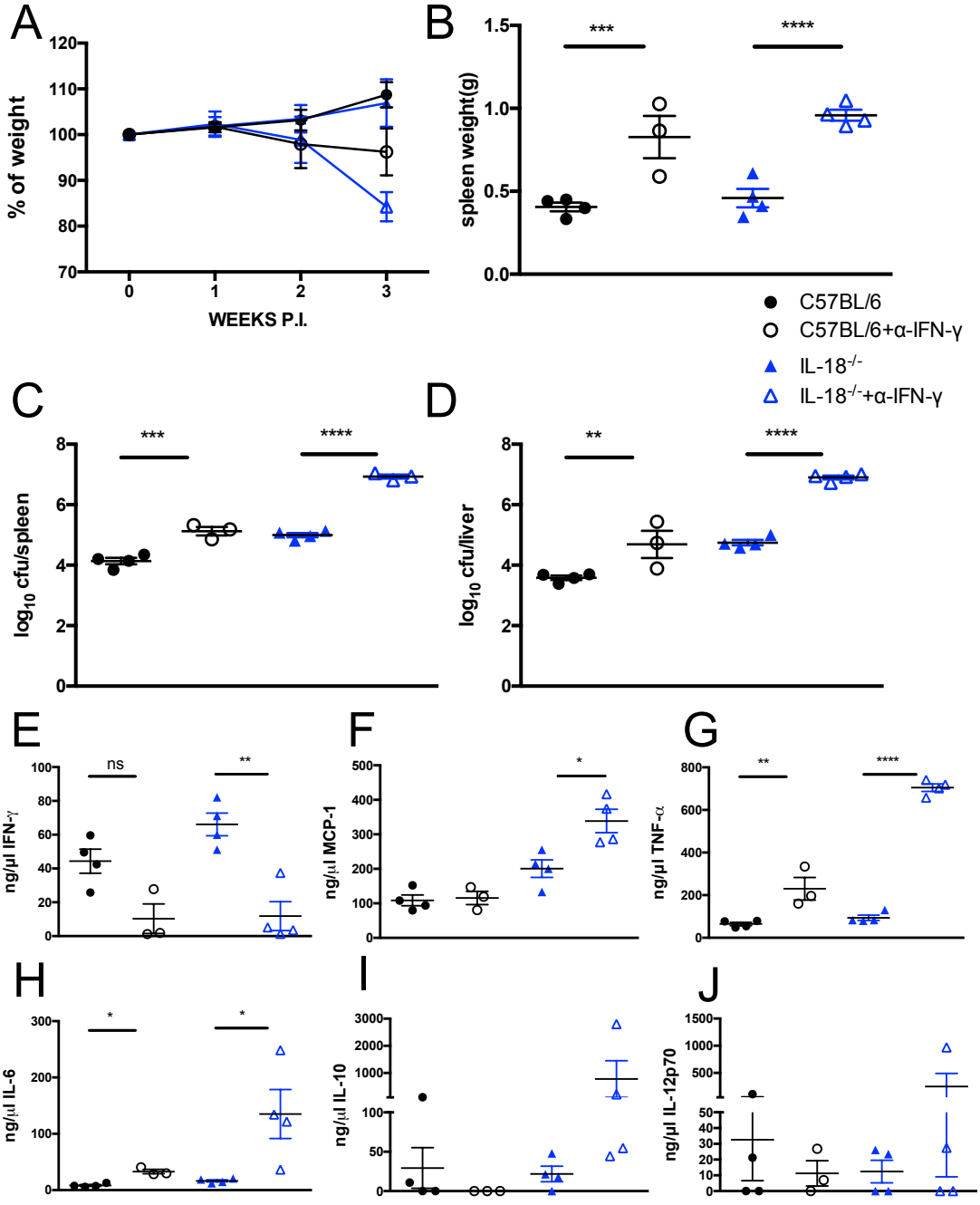

Figure 4-4: Neutralising IFN-γ in IL-18$^{-/-}$ mice impairs the host control of bacteria.

*C57BL/6 and IL-18$^{-/-}$ mice were treated with PBS (solid dots and solid triangles) or 200µg IFN-γ neutralising antibody (HB-170-15) intraperitoneally on day -3, 1, 7, 14 and 18 (open dots and open triangles) and infected with 200cfu BRD509 intravenously on day 0. Mice weights were monitored every week (A) and organs include spleen, liver and blood were taken 21 days after infection. Spleens were weighed to determine the splenomegaly (B). Spleens and livers were homogenised to determine the bacteria counts (C-D) and blood were taken for serum inflammatory cytokine levels, including IFN-γ (E), MCP-1 (F), TNF-α (G), IL-6 (H), IL-10 (I) and IL-12p70 (J). Symbols represent data from individual animals. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was used for statistical analyses. ns p>0.05 *p<0.05 ***p<0.001 ****p<0.0001.*

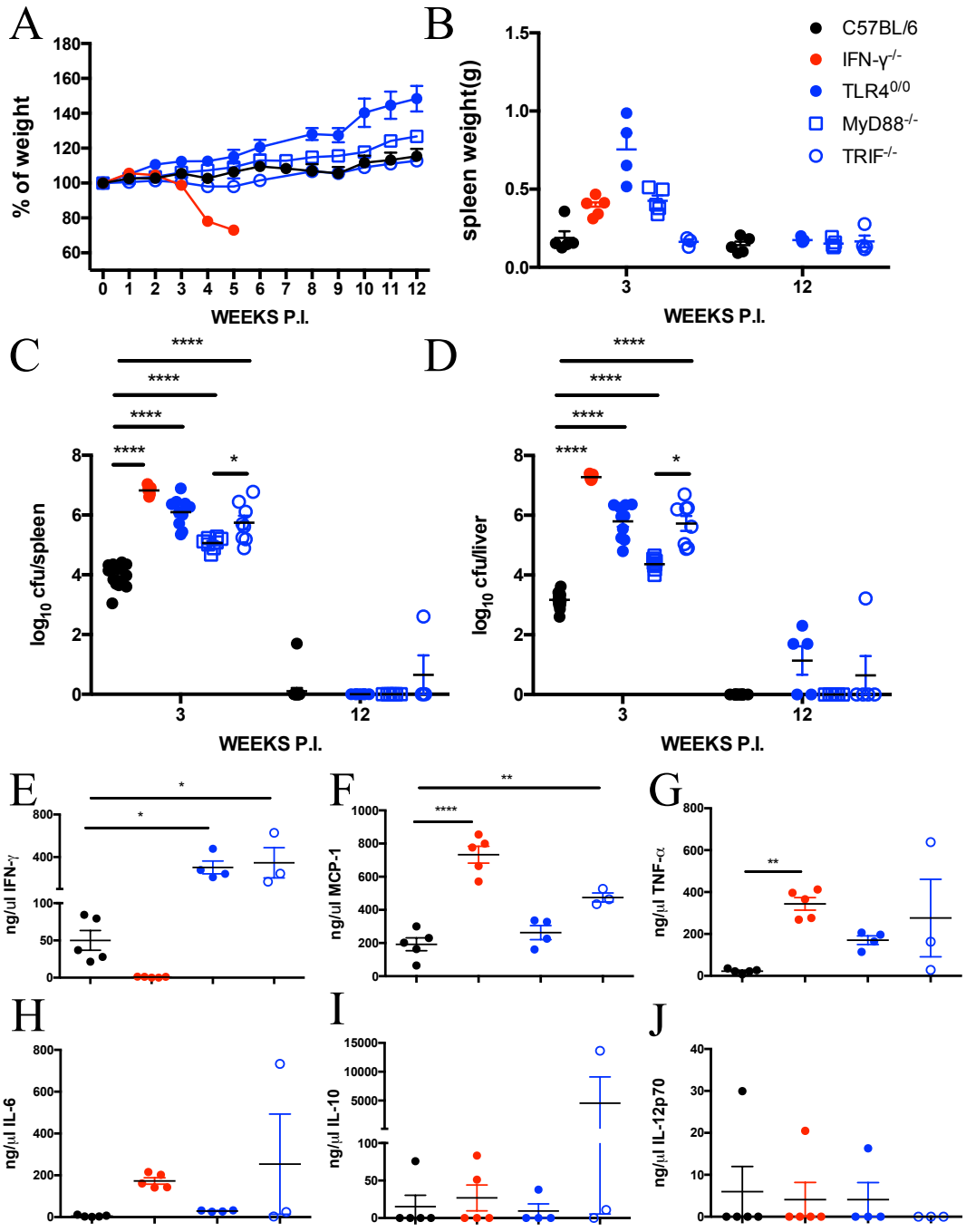

Figure 4-5: TLR4 pathway contributes to early Salmonella control.

*C57BL/6, IFN-γ$^{-/-}$, TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were infected with 200cfu BRD509 intravenously. Mice weights were monitored every week (A) and organs include spleen, liver were taken 3 weeks or 12 weeks after infection. Spleens were weighed to determine the splenomegaly (B). Spleens and livers were homogenised to determine the bacteria counts (C-D) and blood were taken on week 3 for serum inflammatory cytokine levels, including IFN-γ (E), MCP-1 (F), TNF-α (G), IL-6 (H), IL-10 (I) and IL-12p70 (J). Symbols represent data from individual animals. Horizontal lines represent the mean of each group, and error bars show standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was used for statistical analyses. *p<0.05 ***p<0.001 ****p<0.0001.*

4.3 Discussion

These studies followed up on previous findings[71, 152] and extend our understanding of what triggers the rapid IFN-γ burst during *Salmonella* infection. Knockout mice that are deficient in the components of pathways that contribute to IFN-γ secretion were tested using *Salmonella* strain BRD509, a well-studied infection model that requires both IFN-γ-mediated early control of bacterial growth, as well as the induction of adaptive immunity, for eventual bacterial clearance[71].

The NLRC4 inflammasome responds rapidly to *Salmonella* flagellin, and initiates a cascade of responses that leads to the secretion of IFN-γ from NK cells and non-cognate memory $CD8^+$ T cells. Cytosolic flagellin activates the NLRC4 in this process[152]. The studies reported here, both flagellin subunits, FliC and FljB, contribute to the induction of IFN-γ secretion. *S.* Typhimurium deficient in FliC or FljB both triggered IFN-γ secretion 2h after mice were treated with heat-killed bacteria. However the levels of IFN-γ triggered by these two mutant strains are lower than the wild type control, which is possibly due to the expression level of the flagellin as it was shown previously that the secretion of IFN-γ is flagellin dose-dependent[152]. Another explanation might be that both of the flagellin proteins are needed to induce the maximal reaction. FliC and FljB are alternatively expressed flagellin subunits under the control of inversion of the promoter for FljB[175]. When the *fljBA* promoter is in the direction for *fljBA* operon transcription, flagellin FljB is expressed, and also FljA, which is a transcriptional inhibitor of the *fliC* gene. Once the promoter is inverted, FljB or FljA cannot be expressed, thus the flagellin FliC is produced[176]. It is possible that flagellar phase variation contributes to the invasion of *S.* Typhimurium, however, studies to-date have not shown any differences in the triggering of immune responses between the two flagellin proteins, apart from distinct antigenicities conferred through T cell epitopes within the flagellin sequences[177-179]. It was reported that the switch between FliC and FljB does not differentially induce NF-κB through the TLR5 pathway[178] and IL-1β through NLRC-4 pathway[179] and in studies reported here, showed that FliC and FljB appear to be equally capable of inducing IFN-γ through NLRC-4. It is still unclear if flagellar phase variation contributes to bacterial pathogenesis via as yet undiscovered mechanism or is simply an evolutionary redundancy in *Salmonella* to guarantee the functioning of flagella when antibodies against a flagellin type are raised.

Caspase-1 and IL-18 are integral components in the NLRC4 inflammasome pathway, and knockouts in caspase-1 or IL-18 led to the absence of IFN-γ burst within 2 hours of given HK *S.* Typhimurium[152]. This pathway was also shown to be significant in various yeast and bacterial infections including *Cryptococcus neoformans*[249], *Propionibacterium acnes*[250] and *Yersinia enterocolitica*[251]. However caspase-1 or IL-18 knockout mice were able to control and clear infection with *Salmonella* BRD509 despite a reduced systemic level of IFN-γ compared to their WT B6 counterpart (Fig.4-3A). This result indicates that although the NLRC4 inflammasome pathway contributed to the rapid IFN-γ burst, the role for this pathway in the longer-term control or in the clearance of bacteria over an extended infection period is likely limited. Although caspase-1 and IL-18 are in the same signalling pathway, approximately 10-fold differences in the bacterial load, and levels of IFN-γ 2-3 weeks after infection in $ICE^{-/-}$ and $IL\text{-}18^{-/-}$ mice were observed. This could be due to a few facts: Caspase-1 does not only activate IL-18 but also IL-1β, which plays important role in immune control of various bacteria infection, such as *Legionella pneumophila*[252], *Yersinia pestis*[253], *Klebsiella pneumoniae*[254] and *Salmonella* Typhimurium[156]. However, IL-1β can also be processed through caspase-1-independent pathways[255-258], and in $Casp1^{-/-}$ mice the responses of IL-1β to *Mycobacterium tuberculosis*[257] and stimuli that cause inflammation[256, 257] were not different from WT mice. Thus the contribution of the decrease of activated IL-1β in $ICE^{-/-}$ may be limited. Caspase-1 is also involved in the process of pyroptosis and this form of cell death may contribute to the control of bacteria. Also, $ICE^{-/-}$ mice used in this study were also deficient in caspase-11 and the activities of capapse-11 may have contributed to immune protection that led to the difference between $ICE^{-/-}$ and $IL\text{-}18^{-/-}$.

Neutralisation of IFN-γ in $IL\text{-}18^{-/-}$ mice led to a more severe infection and higher bacteria loads compared to the untreated $IL\text{-}18^{-/-}$ mice, suggesting that lower amount of IFN-γ is sufficient to provide protection, i.e. independent to the levels produced via IL-18, from activation of the NLRC4 inflammasome[151]. The bacterial load or the extent of morbidity measured as weight loss was different between B6 treated with anti-IFN-γ antibody, and $IL\text{-}18^{-/-}$ treated group. WT mice are fully capable of secreting IFN-γ, and it is possible that if the IFN-γ antibody did not fully neutralise all of the IFN-γ, the residual IFN-γ still supplied some protection to the host for

controlling *Salmonella* replication. Although every attempt was made to neutralise the cytokine, and to measure its neutralisation, antibody-mediated neutralisation (cf. genetic deletion) may not guarantee full reduction of cytokine through the whole infection period. Other cytokines were also tested to further understand host protection in IL-18$^{-/-}$ mice. MCP-1, TNF-α and IL-6 levels increased with the treatment of IFN-γ antibodies (Fig.4-4F-H), suggesting that these cytokines may be further induced in the absence of IFN-γ, in an attempt to facilitate host defence, although these elevated levels are still insufficient for the controlling the bacteria.

IFN-γ is crucial for host immunity against a number of bacterial infections[141], including *Listeria monocytogenes*, *Mycobacterium* and *Salmonella*[71, 141, 143-145, 259-263]. During *Salmonella* infection, multiple pathways, such as TLR4-mediated IL-12p40[264] and NLRC4-mediated IL-18[265], are able to induce the secretion of IFN-γ in T cells[152] and NK cells[266]. Blocking one of the pathways that result in IFN-γ secretion, e.g. caspase-1/IL-18 seems to have little impact on host immunity (Fig.4-2).

As IL-12p40 regulated by TLR4 pathway is another important source of IFN-γ[159-161, 166], knockout mice that are deficient in the TLR4 pathway were tested using the BRD509 infection model. As reported, TLR4 senses LPS[267-269], activates MyD88[167] or TRIF[168-170], and induces the production of various cytokines, including TNF-α, IL-12p40 and IFN-γ, which all play important roles in host immunity[167, 169, 172, 270, 271]. In these studies reported here, the results show that TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice infected with *Salmonella* developed an infection characterised by a very high bacterial load in the spleen and liver, but were able to ultimately control and clear the bacteria (Fig.4-5A-D). Interestingly, this increased bacterial replication occurred despite a much higher level of IFN-γ in the serum compared to that of wild-type control mice, suggesting some other mechanism other than IFN-γ, mediated via TLR4 pathway, contributed to early control of bacterial growth. Again, this result suggests that the production of IFN-γ is not solely dependent on one pathway, and when one pathway is deficient or blocked, it is likely that compensation from other pathways continue to produce IFN-γ and thus confer sufficient immune protection to the host. The increased bacterial load and serum levels of IFN-γ in TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice confirmed that TLR4 pathway play an important role in defending *Salmonella*

infection, yet this early control of bacteria is likely to be mediated via cytokines other than IFN-γ.

The activation of the TLR4 pathway also leads to the production of TNF-α[272-275], which was shown to be important for the control of *Salmonella* infection in both humans and mice[7, 276-278]. It is usually considered that TNF-α regulation is mediated through the MyD88 pathway[272-274], however, it is also reported that TRIF pathway enhances the translation of TNF-α mRNA[275]. TNF-α and IFN-γ can both lead to the production of iNOS, which has antimicrobial activities in macrophages, to control the growth of *Salmonella*[279]. Thus, it was hypothesised that the increased bacterial level in TLR4$^{0/0}$, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice resulted from the lack of TNF-α from the TLR4 pathway. However, in *Salmonella*-infected TLR4$^{0/0}$ mice, the serum level of TNF-α was higher than in wild type mice (Fig.4-5G). Since this increased amount of TNF-α was not as obvious as the increase in bacterial load, it is probable that TNF-α was responsible for the high bacterial load. Of the two key adaptors of the TLR4 signalling pathway, MyD88 and TRIF, the latter appeared to contribute more in the immune control of *Salmonella* infection, since TRIF$^{-/-}$ mice showed a higher bacterial loads than MyD88$^{-/-}$ mice (Fig.4-5C-D).

The serum levels of IL-6, IL-10 and IL-12p70 were also tested in every mouse strain and it is shown that the serum levels of these cytokines were very low during infection with *Salmonella* under all conditions tested, suggesting these cytokines may not make major contributions toward the control of *Salmonella* infections, if the serum concentrations are proxies for functional levels in infected tissues. However, it needs to be noted that the serum levels of cytokines may not be indicative of the local concentrations in specific sites of infection.

Collectively, although the NLRC4 pathway and the TLR4 pathway were shown to play a role in host immunity during infection with *Salmonella* BRD509, knockout mice that were deficient in either of these two pathways were not as equally immunocomprised as IFN-γ$^{-/-}$ mice in their control of *Salmonella* infections. Although IFN-γ is vital to the immune control of *Salmonella* infection, blocking the source of IFN-γ did not significantly impact gross host immunity, suggesting that the host may only require a small amount of IFN-γ, or that redundancy in provision of the cytokine

is an important feature of resistance to intracellular pathogens. The NLRC4 pathway responds to *Salmonella* rapidly but has little impact over the longer-term control or clearance of the bacteria. Finally, the TLR4 pathway contributes to early control of bacteria but likely through an IFN-γ-independent pathway. Further research in the regulation and the downstream of IFN-γ will help understanding the significance and the mechanisms of where and IFN-γ is involved in resistance, an important element in the re-design of an optimal *Salmonella* vaccine or an immune-mediated treatment for *Salmonella*, where it is functionally antibiotic resistant.

Chapter 5

General Discussion

Infectious diseases caused by pathogenic serovars of *Salmonella enterica* still pose a significant health problem globally. Typhoid fever is endemic in developing countries in Africa, Asia, Latin America and Oceania, causing an estimated disease burden of 27 million cases and 220,000 deaths annually[29]. In addition, non-typhoidal *Salmonella* (NTS) serovars such as Typhimurium and Enteritidis cause 93.8 million cases with 155,000 deaths annually across the world[20]. Recently, an invasive form of NTS (iNTS) has been described and it is particularly prevalent in individuals infected with HIV or malaria, leading to high fatality (~20%) in co-infection settings[51-53].

Two vaccines against *Salmonella* serovar Typhi are currently available, namely the live attenuated vaccine Ty21a and a conjugated vaccine with the Vi capsular polysaccharide[280], but neither afford very strong protection. The efficacies of the vaccines are about 50% (51% for Ty21a vaccine and 55% for Vi vaccine)[281] and the vaccines are not effective in children under 2-5 years of age [281, 282]. Most importantly, these two vaccines only target *S.* Typhi[281-283], thus they cannot be used for protection against NTS and emerging iNTS infections. With the increasing multi-drug resistance in iNTS isolates[284], the effectiveness of antibiotic treatment is become limited and there is an urgent need for developing novel vaccine and treatment strategies against *Salmonella,* especially for iNTS strains where significant disease burden is met with very few effective prophylactic or therapeutic options.

For the rational design of a vaccine or therapeutic treatment for salmonellosis, it is important to understand the host-*Salmonella* interactions, particularly in settings whereby *Salmonella* utilises several mechanisms to invade the host. The failure of the host to generate effective immunity against the bacteria can have dire consequences. As DCs and IFN-γ both play important roles in anti-*Salmonella* immunity in general, and the latter in *Salmonella* infections more specifically, this dissertation describes studies addressing aims to 1) understand the early *Salmonella*-DC interactions, and 2) the contribution of IFN-γ related pathways in the control of experimental *Salmonella* infections.

The immune system can be broadly described in two components, the innate immune system and the adaptive immune system. The innate immunity, is the first line of host defence and consists of cells such as macrophages, DCs and neutrophils[74] . Upon

sensing pathogens through pathogen-recognition receptors, these cells rapidly initiate a cascade of responses that activate antimicrobial pathways that in turn provide co-stimulatory signals for the activation of the adaptive immune system[75-77]. The adaptive immune system generates antigen-specific immune responses after it is triggered by the innate immune system, and can form long-lasting immune memory[74]. It is essential for the innate and adaptive immune systems to coordinate, and DCs play an important role in linking and regulating the two systems[87-95]. In the case of murine *Salmonella* infections, DCs detect *Salmonella* via a variety of PRRs either on the plasma membrane, such as TLR4[167], or in the cytosol, e.g. NLRC4[126-128], and induce antimicrobial responses from other cells, such as NK cells and T cells[90]. Also, DCs produce or induce the production of key cytokines, such as IL-12, IL-18 and IFN-γ to facilitate the bacteria killing[82]. DCs can degrade intracellular *Salmonella* and present peptides for the activation proliferation of T cells via MHC complexes[87-95]. These functions make DCs important in generating immunity against intracellular pathogens[81, 285], intracellular parasites[286] and cancer[287-289].

The importance of DCs in the generation of immunity makes DCs a key target for pathogens seeking to evade immune detection by the host defence[81]. Viruses, such as HIV[290], coronavirus[291] and measles virus[292], utilise molecules expressed on the DCs surface as point-of-entry receptors to invade the immune system. Other viruses, such as EBV, possess the ability to interfere with the antigen presenting function of DCs by increasing expression of IL-10 to polarise specific T cells into a 'less protective' phenotype[293, 294]. A variety of fungi, parasites and bacteria have similar mechanisms to suppress the immune functions in DCs. *Histoplasma*[295], *Leishmania*[296] and *Mycobacterium*[297] are reported to be able to inhibit DCs from producing IL-12 so that the activation of type 1 T helper cells (Th1) is impaired.

To understand the contribution of DCs in immunity against *Salmonella*, it is important to understand how the bacteria interact with the cells. Previous reports have shown that *Salmonella* have evolved mechanisms to interfere with DCs, including down-regulating flagellin[40], regulating phagolysosomal fusion and delaying vacuole acidification to survive inside DCs[38, 39, 104], forming SCV to survive and replicate[102, 103], manipulating the MHC molecules expressed by DCs[112, 113] and causing death in DCs[114, 115].

In Chapter 3 it was demonstrated that *in vivo* infection with *S.* Typhimurium caused a reduction in different subsets of DCs within the tissues examined within one day of infection, and that *in vitro* infection with *S.* Typhimurium caused rapid death in bone marrow-derived DCs within 2 hours post-infection. The level of cell death in DCs correlated with the infection dose, and this rapid death in DCs was shown to involve *Salmonella* virulence factors SPI-1 and flagellin, as *Salmonella* mutant strains that did not express SPI-1 or flagellin showed reduced capacity to cause death in DCs. Using heat-killed *S.* Typhimurium and BMDCs generated from mouse strains, it was also shown that TLR4 and its downstream adaptor TRIF, which respond to extracellular *Salmonella* LPS[159, 166, 167], mediate *Salmonella*-induced death in DCs but most likely via cytokine-independent mechanisms. Furthermore, caspase-1/11 was also shown to participate in the rapid DC death. It is likely that NLRC4 responded to cytosolic *Salmonella* flagellin[152], delivered by SPI-1 encoded T3SS[38, 133, 134], triggering the activation of caspase-1 and caspase-11[53, 54], and leading to pyroptosis in DCs[99, 153]. It is reported that caspase-11 can be non-canonically activated by intracellular LPS, yet it remains controversial if TLR4 and TRIF are required for this activation[173, 241-246]. Thus it is unclear whether TLR4 pathway and NLRC pathway converge or work via independent mechanisms to cause death in DCs.

It is also demonstrated in Chapter 3 that *Salmonella* caused a death in bystander DCs, i.e. DCs that were not directly infected. Death in these bystander DCs could be caused by soluble components released during infection of the DC culture. One possible explanation is that infected DCs release cytotoxic cytokines that cause death in bystander DCs. To test this hypothesis, monensin treatment was used to block protein transport but bacteria-free supernatant from infected DC cultures was still able to induce death in uninfected DCs, showing that cytokines released by DCs may not be the direct cause of DC death. It has been reported that cells undergoing pyroptosis release ASC, an adaptor protein of the NLRC4 inflammasome, which is taken up by neighbouring cells to activate caspase-1, leading to cell death[247]. This phenomenon could, at least in part, explain the observed death bystander DCs.

In summary, Chapter 3 demonstrated that *S.* Typhimurium is able to cause a rapid death in both infected DCs and bystander DCs and this death is mostly likely to be

pyroptosis mediated via SPI-1 and flagellin. The mechanisms involved in the death of *Salmonella*-infected DCs *in vitro* can potentially be exploited as therapeutic targets for generating better immunity against *Salmonella*. However, an important question to ask is whether death in DCs is of benefit to the host or to the bacteria. On one hand, *Salmonella* has developed several mechanisms to interfere with the functions of DCs, such as manipulating MHC expression by DCs[112, 113], suggesting that reduced viability in DCs is correlated with reduced immune surveillance and may promote bacterial survival. On the other hand, killed DCs release intracellular *Salmonella* during the lytic process[298] thereby "expelling" *Salmonella* from a potential replicative niche and exposing the bacterium to killing by surrounding immune cells. Hence, rescuing infected DCs from death may serve as shelter or incubator for *Salmonella* to persist and eventually spread further within the host. To understand better whether protecting DCs from death is beneficial for immunity against *Salmonella*, further experiments are needed to further confirm the mechanisms of the rapid death in infected DCs and bystander DCs. For instance, experiments are needed to make clear of the contribution of caspase-1 and caspase-11 by using Casp-$1^{-/-}$ mice and Casp-$11^{-/-}$ mice. To confirm the contribution of ASC, apply ASC to naïve DC cell to examine if death can be induced and block ASC release or neutralise released ASC in the supernatant to test if the bystander DCs can be rescued from death. By rescuing bystander DCs, it can be tested whether anti-microbial responses can be enhanced.

The production of IFN-γ, as mentions previously, occurs downstream from DC activation[82], and a range of cells including B cells, T cells and NK cells can release IFN-γ[299-304]. Patients with deficiency in IFN-γ receptors IFNGR1 or IFNGR2 show increased susceptibility to intracellular bacterial infections, such as *Mycobacterium bovis* strain Bacillus Calmette-Guerin (BCG)[305-307]. Similarly, it has been shown in mice that in infections with various viruses, parasites and bacteria, e.g. encephalomyelitis virus, *Leishmania major, Toxoplasma gondii, Listeria monocytogenes*, *Mycobacterium* and *Salmonella*, IFN-γ plays a critical part in host defence[71, 141, 143-145, 259-263]. IFN-γ is found to be significant in immunity against cancer[308], possibly through activation of similar defensive mechanisms to those that occur during infection resistance.

IFN-γ participates in multiple immune responses during the host defence, including regulating the trafficking of immune cells via up-regulating chemokines[309, 310] and adhesion molecules[311, 312], activating macrophages[313] and neutrophils[314] to enhance nitric oxide production, tryptophan depletion, and up-regulation of lysosomal enzymes to facilitate bacteria killing[279], protecting macrophages from apoptosis induced by pathogens[315], up-regulating MHC-I and MHC-II to increase antigen presentation[316-318], inducing naïve $CD4^+$ T cell differentiation towards Th1 cells[314, 319, 320], promoting B cell isotype switch[259, 321], etc.

The regulation of IFN-γ production is complicated as a few signalling pathways are involved[152, 157, 160, 161, 164, 173]. One of these pathways, the NLRC4 inflammasome signalling pathway, was reported to respond rapidly to cytosolic *Salmonella* flagellin, inducing pro-inflammatory cytokine IL-18 release, which stimulated IFN-γ secretion in subsets of T cells and NK cells[152]. This study demonstrated that the two variants of flagellin proteins, FliC and FljB, are equally potent in triggering the IFN-γ 'burst' in mice. The two variants are alternatively expressed[176], however the proteins are highly similar in sequence and funtions[180]. It is still unclear what unique role each protein plays in bacterial invasion.

To further explore the contribution of the NLRC4 inflammasome pathway during *Salmonella* infection, studies in Chapter 4 utilised mice that are deficient in caspase-1 or IL-18, and showed that deficiency in the NLRC4 pathway reduced the serum level of IFN-γ significantly, yet this reduction of IFN-γ did not affect the control of bacterial growth or the clearance of bacteria. The total production of IFN-γ was not fully compensated from other IFN-γ regulating pathways, indicating that the NLRC4 pathway is important for very high levels of IFN-γ production, however IFN-γ from other pathways still can provide sufficient protection to the host during *Salmonella* infection.

Another important signalling pathway, the TLR4 pathway, signals through MyD88 or TRIF after sensing LPS, inducing the release of IL-12 that leads to IFN-γ production[322, 323]. It is also shown in this study that deficiency in the components of TLR4 pathway, namely TLR4, MyD88 or TRIF, resulted in significantly increased bacterial load in the infected spleen and liver compared to wild type mice during early

weeks after infection, but that the high load of *Salmonella* was controlled and cleared subsequently. This suggests that while the TLR4 pathway may participate in the early control of *Salmonella*, nevertheless it plays a minimal role in bacterial clearance. The higher IFN-γ levels in the serum of $TLR4^{0/0}$, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice compared to wild type mice after infection suggests that IFN-γ production can be compensated from other signalling pathways and that the failure to control *Salmonella* during early weeks post-infection was not due to the lack of IFN-γ, suggesting that the TLR4 pathway may be involved in other antimicrobial mechanisms. Interestingly, the bacterial load is higher in $TRIF^{-/-}$ than $MyD88^{-/-}$ mice, suggesting that TRIF may play a core significant role in immunity responses against *Salmonella.*

The studies presented in this dissertation showed that the IFN-γ production is not solely dependent on one signalling pathway during *Salmonella* infection. Though IFN-γ is essential to host defence against *Salmonella*[71], and it was proposed as a treatment for intracellular bacterial infection[324], it is demonstrated in this study that deficiency in one IFN-γ regulating signalling pathway or reduced amount of IFN-γ production ultimately did not have much impact on host defence. Further research will be needed to fully understand the regulation and the downstream effect of IFN-γ induction. Understanding the significance and the mechanisms around IFN-γ-mediated immunity will hopefully provide us important insights into how to design better vaccine or treatment strategies for *Salmonella* infection.

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Appendices

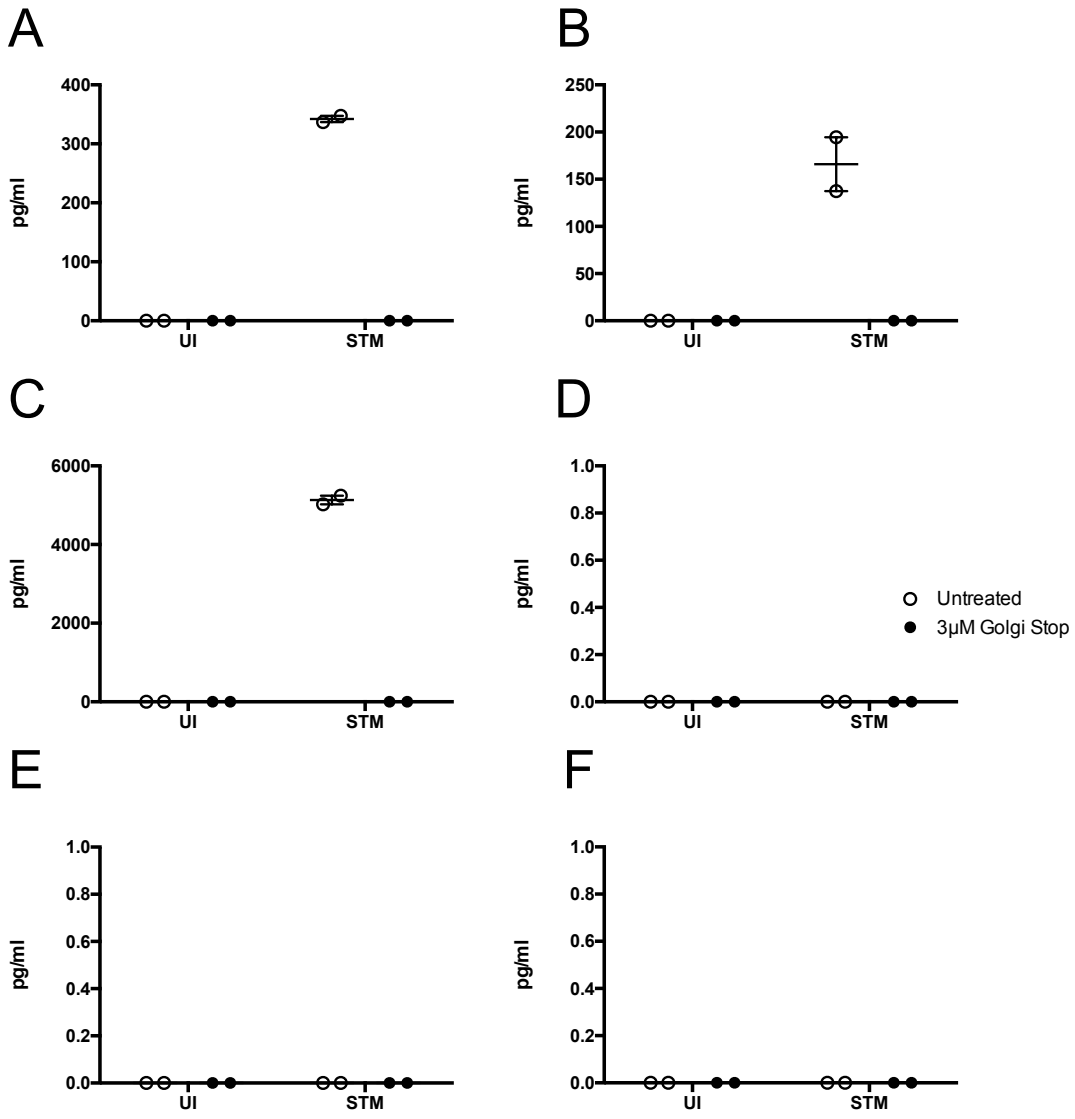

Figure S1: Cytokines were blocked from secreting into supernatant after treatment of monensin.

Monensin was added to DCs at 3mM for 2h prior to the 2 hour infection (MOI 10) of S. Typhimurium SL1344 to stop protein transport, including cytokine secretion. Supernatants were collected to determine the levels of cytokines, including IL-6 (A), MCP-1 (B), TNF-α (C), IFN-γ (D), IL-12p70 (E) and IL-10 (F) by CBA.

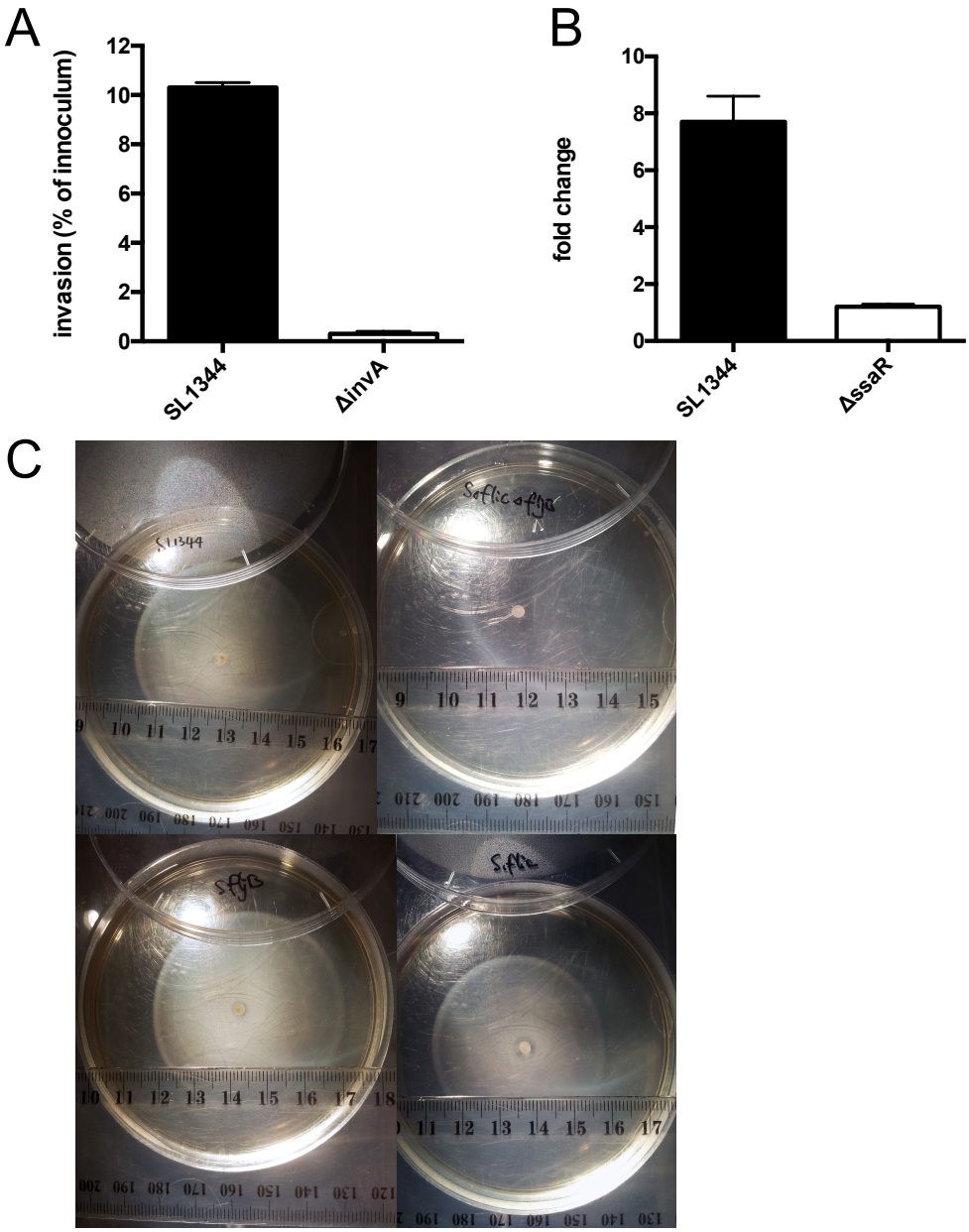

Figure S2: Phenotyping of mutants strains of S. Typhimurium.

MDCK cells were infected with S. Typhimurium SL1344 or ΔinvA at MOI 10 for 2 hours and cells were washed and lysed after infection. Cell lysates were diluted and plated on LB agar plates for bacteria counts (A). Bone marrow derived macrophages were infected with S. Typhimurium SL1344 or ΔssaR at MOI 10. Cells were harvested and lysed at 2h and 24h post infection. Cell lysates were diluted and plated on LB agar plates for bacteria counts. Bacteria counts at 24h were compared to counts at 2h for fold change (B). S. Typhimurium SL1344 , ΔfliC, ΔfljB, and ΔfliCΔfljB were stabbed into 0.3% soft agar and incubated at 37°C for 12 hours. Motility of the bacteria strains were measured (C).

Author/s:
Yang, Chenying

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