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Contributions of the inhibitor of kappa B kinases (IKKs) in macrophages and neutrophils after *Francisella tularensis* live vaccine strain (LVS) infection.

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by

Sylvia Samaniego

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Abstract of the Dissertation

Contributions of the inhibitor of kappa B kinases (IKKs) in macrophages and neutrophils after *Francisella tularensis* live vaccine strain (LVS) infection.

By

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The immune system is a complex network of cells regulated by a number of signaling pathways to drive specific host defenses against invading pathogens. The NF-κB (nuclear factor of the kappa light chain enhancer in B cells) family of inducible transcription factors is a critical regulator of many innate and adaptive immune responses. NF-κB activation by several receptors converges upon two upstream kinases, IKKα and IKKβ, that regulate gene induction. Many studies report that IKKβ is the critical kinase involved in NF-κB activation, although at a transcriptional level, both kinases are needed to obtain the full spectrum of gene induction. More recently, in an *in vivo* bacterial infection model both IKKα and IKKβ were reported to have anti-inflammatory properties through different mechanisms.

The intracellular bacterium *Francisella tularensis* is able to down modulate inflammatory reactions within macrophages and neutrophils to provide a niche for bacterial growth. To this end, I asked whether disruptions of NF-κB signaling, specifically through IKKα and IKKβ, could promote inflammation and allow the host more resistance to infection.

I used conditional gene targeting of IKKα and IKKβ to generate mice with specific deletions for either kinase in these cells. These mice were used in a tularemia infection using the live vaccine strain of *Francisella tularensis* (*Ft.* LVS) as a model to elucidate the specific contributions of each kinase.

Contrary to what has been previously reported for Group B Streptococcus infection models, IKKβ but not IKKα, was required for host resistance and survival in tularemia infection as 100% of *Ft*. LVS infected mice succumbed to lethal infection within 10 days.

In a sublethal model of infection both IKKα and IKKβ contributed in different ways to the maintenance of hepatic granulomas after infection. Depletion of IKKα led to fewer, but sometimes, large necrotic granuloma formation indicating a potential role for IKKα in the clearance of apoptotic cells.

IKKβ depletion resulted disordered granuloma structures and elevated bacterial colonization and growth throughout the infection. This led to increased inflammation as early as 2 days post infection as evidenced by a polarization towards M1 macrophages and IL-12 production. Compensation mechanisms to reduce inflammation such as an increase in myeloid derived suppressor cells or a subsequent M2a macrophage polarization occurred, but were not able to control inflammation or bacterial growth in

these mice. In addition, IKKβ loss resulted in protracted IFN-γ production by cytotoxic T lymphocytes.

Overall, IKKα may be more important in neutrophils for the clearance of apoptotic cells, while IKKβ is required more globally to prevent inflammation and control bacterial colonization that cannot be compensated by anti-inflammatory mechanisms. These functions appear to be correlated with early activation of both macrophages, neutrophils and extrinsic activation of cytotoxic T lymphocytes.

Dedicated to my dear father and son,
Who have nourished my soul
And taught me
Much about love, laughter and adventure.
And for which I am grateful to be
Their eternal student

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List of Commonly Used Abbreviations:

Arg-1	Arginase 1
AKT	viral-Akt murine thymoma viral oncogene homolog
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
Bcl-3	B-cell leukemia/lymphoma-3
CHUK	Conserved Helix Loop Helix Ubiquitous Kinase (IKKα/IKK1)
CFU	Colony Forming Units
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
d.p.i.	Days post infection
FcR	Fragment, crystallizable receptor
FIZZ1	Found in inflammatory zone 1
Ft.	Fransicella tularensis
GBS	Group B *Streptococcus*
HSP60	Heat shock protein 60
ICAM	Intercellular cell adhesion molecule
i.d.	intradermal
igl	intramacrophage growth locus
i.n.	intranasal
i.p.	intraperitoneal
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL-1β	Interleukin 1, beta
IL-1R	Interleukin 1 receptor
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15

iNOS/NOS2	inducible Nitric Oxide Synthase
IκBα	Inhibitor of NF-κB, subunit alpha
IkB	Inhibitor of the kappa light chain enhancer in B cells
IKK	Inhibitor of the kappa light chain enhancer in B cells kinase
IKKα	IkB kinase subunit alpha (also known as IKK1/CHUK)
IKKβ	IkB kinase subunit beta (also known as IKK2)
IKKγ	IkB kinase subunit gamma (see NEMO)
IRAK	IL-1 receptor associated kinase
Irf1	Interferon regulatory factor 1
KC	1. Mouse homolog of CXCL1
	2. Kupffer cell
LPS	Lipopolysaccharide
LD_{50}	Lethal dose of ½ the population
LVS	Live Vaccine Strain
LTβ	Lymphotoxin Beta
LysM	Lysozome, M
Mal	MyD88 adaptor-like; TIRAP
M-CSF	Macrophage colony stimulating factor
MCP1	Monocyte chemotactic protein 1
MDSC	Myeloid derived suppressor cell
MHCI	Major Histocompatibility Complex I
MHCII	Major Histocompatibility Complex II
MR	Mannose receptor
Mip2	Macrophage inflammatory protein 2, CXCL2
MOI	Multiplicity Of Infection
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB Essential Modulator, IKAP1, IKKγ
NF-κB	Nuclear factor of the kappa light chain enhancer in B cells
NIK	NF-κB inducing kinase
NK	Natural killer
NLR	Nod-like receptor
NLS	Nuclear Localization Signal
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PEMS	Peritoneal elicited macrophages
PIAS1	Protein inhibitor of activated STAT1
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein kinase C
PMN	Polymorphonuclear cell
PRR	Pattern recognition receptor

RANKL	Receptor activator of NF-κB ligand
RANTES	Regulated upon activation, normally T-expressed and presumably secreted
Rel	Relish
Relmα	see FIZZ1
RLR	RIG-1-like receptors
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
s.c.	sub cutaneous
SCID	Severe combined immunodeficiency
spp.	species
STAT	Signal Transducer and Activator of Transcription
SUMO	Small ubiquitin related modifier
TCR	T cell receptor
TGFβ	Transforming growth factor, beta
T_H1	T helper cell 1
T_H2	T helper cell II
TIR	Toll/Interleukin receptor domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor family
TNF	Tumor necrosis factor superfamily
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor family
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	Wild type
Ym1	Chitinase like enzyme

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Chapter 1
What is NF-kB?

NF-κB (Nuclear factor of the kappa light chain enhancer in B cells) was discovered over 20 years ago as a nuclear factor binding DNA sequences within the kappa light chain enhancer (1,2). Today, an entire field of research has emerged from this seminal work and is dedicated to understanding the complexities and intricate nature of this family of transcription factors, including its role in common diseases such as cancer, Crohn's disease, inflammation, sepsis, arthritis, autoimmunity and heart disease.

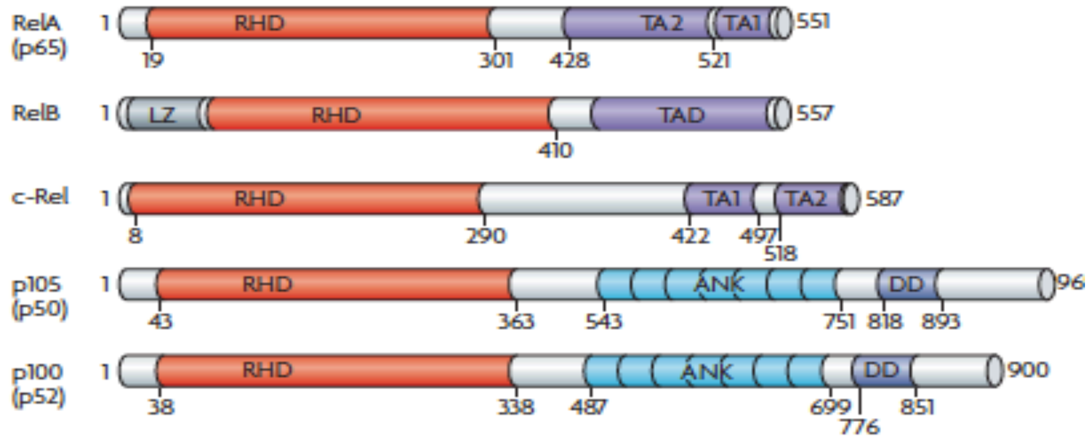
NF-κB transcriptional responses are seen in a number of vital processes including immunity, inflammation, development, cell growth and apoptosis. It is important to keep in mind the delicate balance nature has imposed by incorporating such a ubiquitous pathway to maintain these vital processes and homeostasis.

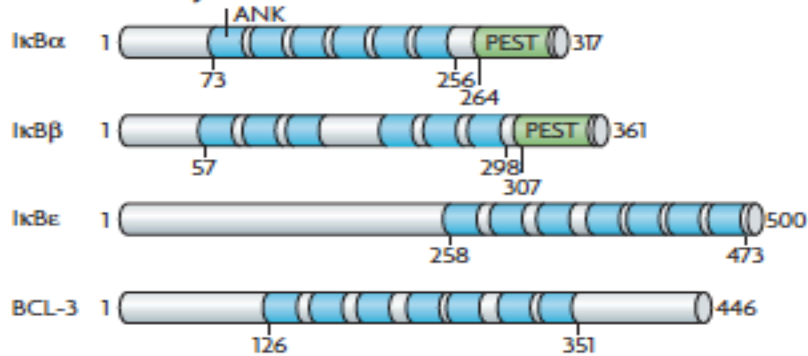
Chapter 1.2
NF-κB Players and Activation

The NF-κB/Rel family of transcription factors consists of five structurally related subunits, which combine to form homo- or heterodimers (Figure 1A). These transcription factors, RelA/p65, c-Rel, RelB, p100/p52 and p105/p50, all contain an

N-terminal Rel Homology Domain (RHD), an Ig-like (immunoglobulin-like) structure involved in DNA binding and dimerization. The RelA/p65, c-Rel and RelB proteins also contain a C-terminal transactivation domain which is required for transcriptional activation, while p100 and p105 precursor proteins contain a series of ankyrin repeats which are similar to the inhibitory domains found in the IκB (inhibitor of Kappa B) protein family.

Figure 1





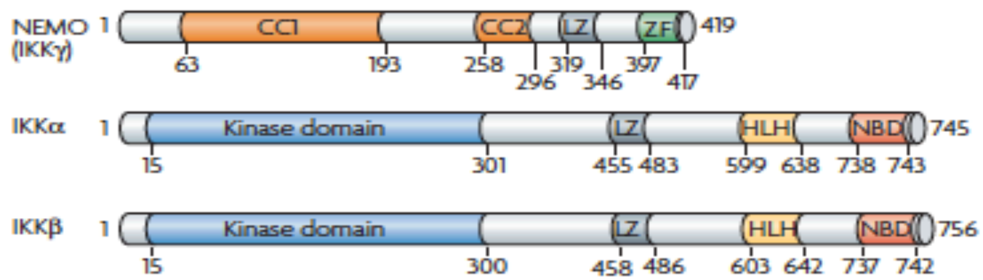


Figure 1: The NF-κB, IκB and IKK families.

A. In mammalian cells, there are five nuclear factor (NF)-κB family members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). p50 and p52 (not shown) are derived from the longer precursor proteins p105 and p100, respectively. All NF-κB family members contain an N-terminal Rel-homology domain (RHD) that mediates DNA binding and dimerization and contains the nuclear-localization domain. The Rel subfamily, RelA, RelB and c-Rel, contain unrelated C-terminal transcriptional activation domains (TADs). TA1 and TA2 are subdomains of the RelA transactivation domain. **B.** The inhibitor of NF-κB (IκB) family consists of IκBα, IκBβ, IκBε and BCL-3. Like p105 and p100, the IκB proteins contain ankyrin-repeat motifs (ANK) in their C termini. **C.** The three core subunits of the IκB kinase (IKK) complex are shown: the catalytic subunits IKKα and IKKβ and the regulatory subunit called the NF-κB essential modifier (NEMO, also known as IKKγ). The principal structural motifs of each protein are shown, together with amino-acid numbers corresponding to the human proteins, although some definitions of where a domain begins and ends might differ between publications. CC, coiled-coil; DD, region with homology to a death domain; HLH, helix–loop–helix; LZ leucine zipper, RelB-transactivation-domain containing a putative leucine-zipper-like motif; NBD, NEMO-binding domain; PEST, domain rich in proline (P), glutamate (E), serine (S) and threonine (T); ZF, zinc-finger domain.

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NF-κB is an inducible system of ready-made transcription factors. In a quiescent state, NF-κB is located in the cytoplasm in association with its inhibitor. Signals that induce activation remove the inhibitor causing NF-κB to translocate to the nucleus to mediate transcription of target genes. This inducible mechanism forgoes the need to synthesize the transcription factor itself and allows for immediate transcriptional responses by the cell. For this reason, the system must be tightly regulated to prevent inappropriate activation.

Inhibitory proteins of the IκB (Inhibitor of Kappa B) family (4,5) (reviewed in (6) and shown in Figure 1B) prevent nuclear translocation by masking the nuclear localization signal (NLS) of NF-κB. Upon receptor activation, signals from these receptors converge upon a multi-protein cytoplasmic complex termed the signalosome. The signalosome, made up of the IKK (Inhibitor of Kappa-B Kinase) proteins (Figure 1C) phosphorylates the NF-κB inhibitor. Phosphorylation promotes the subsequent ubiquitylation and proteosomal degradation of IκB, allowing free NF-κB to translocate to the nucleus. In the nucleus, NF-κB dimers bind a 9-11 bp DNA consensus sequence, called a κB site, located within target gene promoter regions. These κB sites have the nucleotide sequence 5'-GGGRNNYYCC-3' (where R = purines A or G and Y = pyrimidines C or T, and N = any nucleotide).

Chapter 1.3:
The canonical and non-canonical NF-κB pathways

Broadly speaking, two main NF-κB pathways, the canonical/classical and non-canonical/alternative pathways, have been described (Figure 2). The key distinguishing features between these pathways are 1) the types of activating signals, 2) the composition of NF-κB dimers 3) gene outputs and 4) composition of signalosome and regulation of inhibitory proteins.

Classical NF-κB is activated through several different receptor/signaling pathways including TNFR (Tumor Necrosis Factor Receptor superfamily), IL-1R/TLR (Interleukin-1 and Toll-like receptor families respectively), B and T cell receptors, a multitude of cytokine receptors, physiological conditions, stress-related pathways, apoptotic mediators, mitogens and hormones, as well as biological agents such as bacteria, fungi, parasites, viruses and their byproducts (Figure 2, left). These stimuli cause nuclear accumulation of RelA or c-Rel containing dimers, with RelA/p50 being the archetypal NF-κB dimer. A wide array of genes are induced by this pathway including numerous cytokines and their regulators, immunoregulatory genes, cell adhesion molecules, receptors, genes involved in stress response and apoptosis. A selected list of target genes is listed in Table 1 at the end of this chapter (for further information, please also refer to the website www.NF-KB.org).

Figure 2

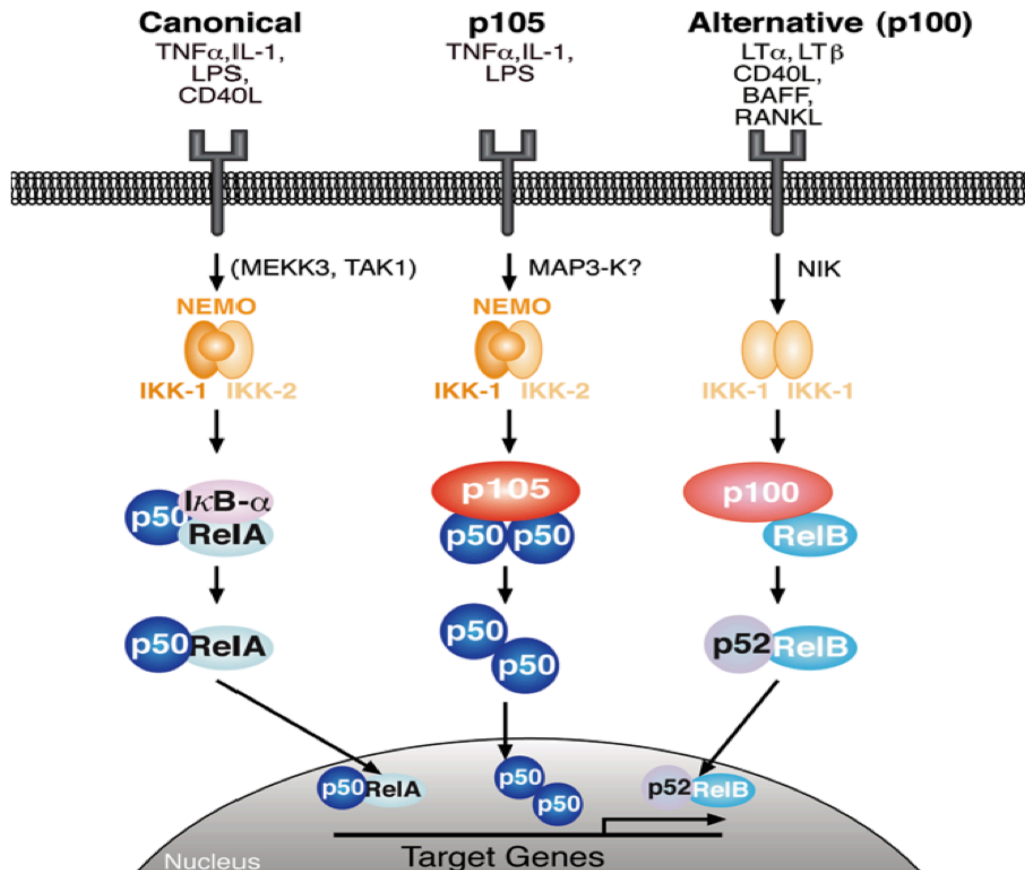

Figure 2: Canonical and Non-Canonical/Alternative NF-κB Pathways
The two main pathways of NF-κB signaling are the Canonical and Non Canonical/Alternative pathways. The canonical pathway (left) is activated by a large number of stimuli to activate target genes involved in inflammation, immunity and cell survival. The p105 (center) pathway is also activated under canonical conditions. The Alternative pathway (right) is more limited in terms of activating stimuli and is involved in secondary lymphorganogenesis and B cell development.

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The p105 pathway (Figure 2, center) regulates the release of mature p50 in both resting and activated cells. It is considered a minor side pathway that is activated by a similar set of canonical activators. In resting cells, p105 undergoes constitutive, but partial proteolytic degradation to remove its inhibitory c-terminal ankyrin domain. Under stimulatory conditions, p105 undergoes a complete but accelerated degradation whereby associated p50 homodimers, p50/p50, are subsequently released (8). Since p50 contains a DNA binding domain but no transcriptional activation domain, p50 homodimers are thought to be repressive in nature (9-12).

The repressive effect of the p50/p50 homodimer is exemplified in the case of LPS-mediated (lipopolysaccharide, a component of Gram negative bacterial cell wall) tolerance (13,14). LPS is a well-studied activator of canonical NF-κB target genes. In macrophages, LPS induces the transcription of proinflammatory cytokines such as TNF-α (Tumor necrosis factor α) and IL-1β (Interleukin-1 β), monocyte/macrophage chemoattractants such as CCL3, 4 and 8 (CC motif ligands 3, 4 and 8), neutrophil chemoattractants such as CXCL1, 2 and 8 (CXC motif, ligand), as well as a host of other genes involved in stress response, apoptosis, adhesion, growth and cell cycle (15). The cell, however, must have a mechanism to regulate this process, as excessive inflammation can lead to tissue damage. "Promoter tolerance" is one mechanism employed by cells to actively and specifically reduce transcription of inflammatory genes. LPS stimulation signals through the canonical NF-κB pathway. At the nuclear level, RelA- or c-Rel-containing dimers are recruited to κB sites located in the promoter region of target genes to activate transcription. After this initial phase, some κB sites undergo a dimer exchange, from RelA- or c-Rel-containing dimers to p50 homodimeric complexes.

Binding of p50 homodimers prevents further gene activation at certain gene promoters and renders the promoters unresponsive to restimulation. In LPS stimulated macrophages, the TNF-α, gene promoter exhibits LPS tolerance. It is exhibited by increased p50/p50 promoter occupation, reduced RNA Pol II (RNA polymerase II) recruitment and down modulation of TNF-α. Furthermore, $p50^{-/-}$ derived macrophages show an increased proinflammatory cytokine profile, including TNF-α (13,14).

The repressive function of the p50 homodimeric complex seems to be dependent on a stabilization mechanism through interaction with Bcl-3 (B cell leukemia/lymphoma) (16). Bcl-3 is a member of the IκB family of proteins, but is unusual in that it is predominantly nuclear rather than cytoplasmic (17). LPS tolerance requires both Bcl-3 and p50 expression, in that loss of expression of either protein results in a hyperinflammatory state. Furthermore, loss of tolerance in the absence of Bcl-3 promotes the polyubiquitylation of and concomitant loss of p50 homodimers from gene promoters of *TNF-α* and *CXCL2* (16,18).

The tolerance reaction is specific, as it does not occur at all primary LPS-activated promoters. Output from genes such as *p50*, *TNFRII* (Tumor necrosis factor receptor II) and *IL-10* (Interleukin-10) are actually increased after rechallenge and may serve to dampen the inflammatory response (13).

However, the p50 homodimer is not strictly repressive. Early experiments using CAT (Chloramphenicol acetyl transferase) and luciferase transcription reporter assays showed that p50 homodimers interact with Bcl3 to activate transcription as well. (19,20). More recently, the p50 homodimer was also shown to interact with CBP (Creb binding

protein) to activate transcription of an IL-10 (Interleukin 10) luciferase reporter construct (21).

The alternative NF-κB pathway (Figure 2, right) seems to be more restricted in terms of activator/receptor pairs and gene induction. Few known ligand signals such as lymphotoxin-Beta (LTβ) (22,23), B cell Activating Factor (BAFF) (24), CD40L (25) and CD70 (26) have been reported to activate this pathway and genes induced by this pathway are limited to secondary lymphoid organogenesis and B cell maturation. RelB/p100 is considered the prototypical NF-κB dimer of this pathway. Unique among all NF-κB subunits, RelB is regulated by p100 and essentially no other IκB proteins (27). The p100 subunit, like p105, also contains ankyrin repeats which maintains its cytoplasmic retention. Ligand activation of the alternative pathway induces IKKα-mediated phophorylation of p100. This "molecular tag" signals it for partial proteolytic degradation and results in the removal of its ankyrin domains, yielding the mature p52 subunit. Once relieved of its inhibitory domain, mature RelB/p52 dimers are free to enter the nucleus and activate gene transcription.

In addition to activation by different ligand and receptor sets and NF-κB dimers, these two pathways differ in signalosome components and activate different gene subsets. The canonical signalosome consists of two kinases, IKKα and IKKβ, and a non-catalytic structural component NEMO/IKAP1/IKKγ (*NF-κB E*ssential *Mo*dulator). IKKα and IKKβ are two closely related kinases exhibiting 52% sequence homology. Each contains an amino-terminal catalytic domain with an ATP-binding residue at lysine 44 and two serine phosphorylation sites (176 and 180, or 177 and 181 for IKKα and IKKβ respectively), which are necessary for kinase function. Other structural motifs contained

within these kinases include a leucine zipper (LZ), important for dimerization, as well as a C-terminal helix-loop-helix (HLH) domain (28-30) and NEMO binding domain (NBD) (31), both of which are important in protein-protein interactions to help regulate kinase activity (30).

IKKα, also known as CHUK (Conserved Helix-Loop-Helix Ubiquitous Kinase), was first cloned by our group (28) in 1995 but its function as an NF-κB regulator was not immediately known. It was later defined as a cytokine responsive kinase of NF-κB (29). Shortly thereafter, the IKKβ subunit was also cloned and together with IKKα was described to make up what was referred to as the IKK signalosome (30). In vitro, both IKKα and IKKβ can phosphorylate the NF-κB inhibitor IκBα (30). However, genetic studies have shown that in vivo, IKKβ is the essential kinase required for IκB degradation and IKKα is largely dispensable.

The third signalosome protein, NEMO/IKAP1/IKKγ, is a structurally unrelated but essential subunit of the complex (32-34). Its carboxy-terminal domain is required for NF-κB activation and it is thought to mainly function as a non-catalytic scaffolding protein. NEMO also aids in the recruitment of upstream serine kinases from several upstream signaling pathways, such as MAPKKK/MAP3K (Mitogen-activated protein Kinase Kinase Kinase-like) proteins including NIK (NF-κB Inducing Kinase), MEKK (Mitogen activated/Extracellular signal-related kinase kinase), TAK (Transforming growth factor β-activated kinase), and Tank Binding Kinase (TBK) to converge upon and activate the signalosome (32-34).

The signalosome in the alternative pathway is, so far, only known to contain IKKα, but not IKKβ or NEMO. NIK is the upstream kinase that preferentially phosphorylates IKKα and was shown to be required for this pathway (35).

Chapter 1.4: The Signalosome Kinases: IKKα and IKKβ Regulation of NF-κB

IKKα$^{-/-}$ transgenic mice develop to term, but die within 30 minutes of birth. Embryos exhibit abnormal limb development and skin defects including lack of epidermal differentiation (36,37). Fibroblasts derived from IKKα$^{-/-}$ animals show proper translocation of RelA to the nucleus after Tumor Necrosis Factor alpha (TNF-α) treatment, indicating that defects are not due to IκB degradation. The latter study also showed reduced TNF-α induced mRNA transcription of *IκBα*, *M-CSF* (macrophage colony stimulating factor) and *IL-6* genes in IKKα knock out mouse embryonic fibroblasts (MEFS). This was an early indication that IKKα has roles in canonical activation exclusive of IκB degradation (38). Transgenic mice deleted for IKKβ, on the other hand, die between embryonic days E12.5-13.5 due to severe liver apoptosis. Lethality was rescued by crossing IKKβ$^{-/-}$ mice with TNFR1$^{-/-}$ mice suggesting that liver apoptosis was likely due to TNF-α toxicity (39). Although biochemically IKKα and IKKβ both phosphorylate IκBα, these studies revealed that IKKβ, not IKKα, was the main kinase required for IκB degradation in canonical signaling. Yet, the fact that some canonical target genes such as IκBα were not fully activated in the absence of IKKα left the question: What exactly is the function of IKKα in the signalosome and/or NF-κB regulation as a whole?

Chapter 1.5
The promiscuity of IKKα

A decade of research has shed some light on these questions. Overall, IKKα is less discriminate when it comes to phosphorylation targets. It can regulate NF-κB activation both in the nucleus and in the cytoplasm, interacting with repressors, coactivators, and NF-κB transcription factors.

Indications that IKKα functions in the nucleus came about from a kinetic study of NF-κB signaling molecules. This study revealed that IKKα and NIK, an activating kinase that preferentially phosphorylates IKKα, shuttle and accumulate in the nucleus after treatment with the CRM1 (Required for Chromosomal Region Maintenance) nuclear exportin inhibitor Leptomycin B. Furthermore, a K44 (lysine 44) kinase-dead mutant of IKKα does not shuttle, suggesting that IKKα's kinase activity is required for this process (40). In addition, a microarray study from our laboratory determined that IKKα is required to yield the full spectrum of gene activation in response to TNF-α and IL-1 (41).

These results combined with results of the knockout studies led others to investigate the possibility that IKKα has a unique role regulating transcription in the canonical NF-κB pathway. Two independent laboratories simultaneously reported a nuclear role for IKKα in response to TNF-α (42,43). Both groups reported an essential requirement for IKKα in the phosphorylation of serine 10 on histone H3, a pre-requisite for GCN (General Control of Amino Acid Synthesis) mediated acetyltransferase activity on lysine 14, a modification associated with gene transcription. Anest *et al.* (28) found

these modifications at the IκBα and IL-6 promoters while Yamamoto *et al.* (27) found similar requirements at IκBα and IL-8 promoters. The latter study also described an interaction and recruitment of IKKα with RelA and CBP (CREB Binding Protein) to NF-κB promoters. CBP is a protein with known histone acetyltransferase (HAT) activity, whereby acetylation serves as a signal to nucleate transcriptional activators and mediate gene upregulation.

Other examples of IKKα mediated gene activation include the IKKα dependent phosphorylation of the SMRT (Silencing Mediator of Retinoid and Thyroid receptors) corepressor complex. SMRT phosphorylation coincides with removal of HDAC3 (Histone Deacetylase 3) and nuclear export of SMRT. SMRT "derepression" is required for RelA mediated transcription at the *IL-8* and *cIAP2* (apoptosis inhibitor 2) genes and cell survival (44). Additionally, IKKα can directly phosphorylate and activate RelA in response to TNF-α and IL-1 mediated through the PI3K/AKT, pathway (45-48).

Interestingly, in addition to its roles in gene activation, IKKα is also reported to be involved in gene repression. IKKα was found to limit inflammation by inhibiting RelA promoter binding in Raw264 macrophages via phophorylation of serine 90 (S90) on PIAS1 (protein inhibitor of activated STAT1). PIAS1 is an E3 SUMO (small ubiquitin-related modifier) ligase that blocks transcriptional activation of STAT1 (signal transducer of activator of transcription 1) and NF-κB transcription factors. The S90 phosphorylation of PIAS1 is required for the repression of NF-κB target genes such as *TNF-α*, *Mip2* (macrophage inflammatory protein 2) and *Irf1* (interferon regulatory factor 1) in response to TNF-α stimulation (49).

The preceding examples demonstrate a clear nuclear role for IKKα in the canonical pathway. However, the cytoplasmic role of IKKα in the canonical signalosome has not yet been determined. Moreover, cross activation between canonical and non-canonical signaling adds to the complexity in teasing apart its role. Recently, a mathematical modeling analysis of NF-κB found crosstalk exists between canonical and alternative pathways. Studies using the non-canonical activator, LTβ (Lymphotoxin Beta), showed that the archetypal canonical NF-κB heterodimer, RelA/p50, is activated in the absence of IκB and this activation occurs via IKKα−dependent p100 processing. Biochemical studies showed that immunodepletion or siRNA (short interfering RNA) targeting of the non-canonical inhibitor p100 increased RelA/p50 binding activity in nuclear extracts from $I\kappa B^{-/-}$ cells (50). It is thought that the kinetics of alternative activation is slower and longer lasting. Perhaps, then, crosstalk is a way to activate some canonical target genes at specific times and for a specific duration without necessitating full canonical activation. In this way it shapes specific functions within the same set of transcriptional pathways.

Chapter 1.6
A matter of context: the IKKs *in vivo*

The ability of a non-canonical stimulus such as LTβ to activate "canonical" NF-κB is a good example of the complexity of this system and highlights that there is still much to be learned. In fact, much of the aforementioned work has been elucidated only through biochemical and/or *in vitro* culture systems. The advent of gene targeting technology has made it possible to examine NF-κB signaling in more detail *in vivo* and this has brought to light some additional complexities. Both IKKα and IKKβ, in addition

to their roles in the proinflammatory canonical signaling pathway, were also found to have anti-inflammatory roles and therefore the roles of IKKα and IKKβ *in vivo* may be context dependent.

IKKα was shown to limit the activation of NF-κB in an *in vivo* Group B *Streptococcus* (GBS) infection model. The mouse model used in this study encompassed a mutant of IKKα where serine residues 176 and 180 are mutated to alanine ($ikk\alpha^{SS/AA}$). In this way, IKKα cannot be phosphorylated. Mutant mice exposed to systemic GBS challenge initially showed lower bacterial titres at 4 hours post infection relative to wild type counterparts. Yet, despite increased clearance, these mice succumbed to infection more rapidly. Furthermore, an increase in neutrophil recruitment and inflammation in lung tissue was also seen. Mice were also tested using the TLR4 (Toll-like Receptor) agonist *E. coli* (*Escherichia coli*) LPS and the TLR2 agonist Zymosan A (a yeast cell wall protein-carbohydrate complex) to see if this was a generalized mechanism. Analysis of lung and liver tissue showed increases in mRNA of inflammatory cytokines, increased leukocyte recruitment and a similar susceptibility to septic shock in response to these agonists as was seen in the GBS infections, indicative of a general mechanism. Radiation chimera models were used to determine that the increases seen were a result of IKKα dysfunction in the hematopoietic lineage, and further analysis showed that $ikk\alpha^{SS/AA}$ macrophages had increased bacterial uptake and inflammatory profile as well. The authors concluded that IKKα acts as a negative regulator of macrophage activation. It was proposed that this may be due to the rate of turnover of RelA and cRel containing NF-κB dimers from promoters because compared to WT animals, $ikk\alpha^{SS/AA}$ mutants showed a delay in promoter clearance (51).

A similar study investigating the role of IKKα in macrophage function came to nearly similar conclusions. In this study macrophages were derived from embryonic livers of IKKα null mice (IKKα$^{-/-}$) and results showed that loss of IKKα had functional consequences as well. These macrophages were better able to take up bacteria and induced increased T cell proliferation, a true test of functionality of the system as this only occurs after proper antigen processing and presentation to T cells. LPS treatment of these macrophages also yielded elevated inflammatory cytokines and chemokines. These increases were not due to increased IKKβ expression and phosphorylation nor were they due to increased RelA phosphorylation of serine 536, a molecular signature associated with transcriptional activation. However, this study showed that although IκBα mRNA levels were increased, protein levels of IκBα were lower. Pulse chase experiments showed that IKKα$^{-/-}$ macrophages were more efficient at IκB degradation and this was likely the reason for the increased proinflammatory state. Furthermore, the authors did not find RelA or cRel turnover as reported in the previous study by Lawrence *et al*. (51). The authors therefore concluded that the increased inflammatory state was likely due to differences in signalosome components between the two model systems; whereas a signalosome in *ikkα*$^{SS/AA}$ animals would likely be complexed with IKKβ, IKKα$^{-/-}$ mice would contain signalosomes only composed of IKKβ dimers which they suggested might be more efficient at IκBα turnover (38).

One of the biggest lessons in context dependency came about in a recent discovery that IKKβ has an anti-inflammatory function *in vivo*. Using mice conditionally deleted for IKKβ (IKKβ cKO) in macrophages and neutrophils, Fong *et al.* (52) found several surprising results. First, loss of IKKβ resulted in a survival advantage of mice in

a pulmonary GBS infection model. This was unexpected as IKKβ was always considered a master regulator of NF-κB proinflammatory transcriptional target genes in response to inflammatory stimuli due to its role in IκB degradation. Furthermore, infection increased expression of IL-12, iNOS2 (inducible nitrous oxide synthase) and MHCII in knock out macrophages. These results indicated that loss of IKKβ polarized macrophages to the inflammatory classical subtype of M1 macrophages (see Table 5 and macrophage discussion in Chapter 3). Airway infection with GBS resulted in a decreased amount of bacteria in BAL (Bronchoalveolar lavage) fluid at 4 hours post infection as well as increased neutrophil tissue infiltration. Despite this seemingly better initial control of infection, resolution was delayed compared to wild type animals. In addition, in vitro assays with IKKβ cKO macrophages showed increased bacterial killing, whereas neutrophils derived from the same animals did not. The authors concluded that increased phagocytosis and bacterial killing were likely due to increased macrophage-mediated neutrophil recruitment and increased iNOS production and not due to increased neutrophil activation (52).

Table 1: Selected NF-κB target genes[a]:

Type		NF-κb target	Alternate names
Cytokines:			
	Interferons:		
		IFN-β	Interferon, fibroblast
		IFN-γ	Type II interferon
	Interleukins:		
		IL-1α	
		IL-1β	
		IL-1Ra	IL-1R antagonist
		IL-2	T-cell growth factor
		IL-6	Interferon, beta-2
		IL-8	CXCL8, monocyte-derived neutrophil chemotactic factor
		IL-9	T-cell/Mast cell growth factor
		IL-10	Cytokine synthesis inhibitory factor, CSIF
		IL-11	
		IL-12A	p35
		IL-12B	p40
		IL-13	
		IL-15*	
		IL-17	
		IL-23A	p19
		IL-27	p28
		IL-27B	EBI3
	TNF family:		
		LT-α	Lymphotoxin-alpha
		LT-β	Lymphotoxin-beta
		TNF-α	Tumor necrosis factor-alpha
		TNF-β	Tumor necrosis factor-beta
		TRAIL	TNFSF10, TNF related apoptosis inducing ligand
	Other:		
		BAFF	B-cell activating factor
		ICOS*	Inducible T-cell costimulator
		MIG	Monokine induced by IFN-γ
		CD154	CD40L

Type		NF-κb target	Alternate names
Chemokines:			
	CC chemokines:		
		CCL1	T cell activation gene 3
		CCL2	MCP-1
		CCL3	MIP-1α, macrophage inflammatory protein 1-alpha
		CCL4	MIP-1β, macrophage inflammatory protein 1-beta
		CCL5	RANTES, Regulated upon activation, normally T-expressed, and presumably secreted
		CCL11	Eotaxin
		CCL15	MIP-5, macrophage inflammatory protein 5
		CCL15	Leukotactin
		CCL17	Thymus and activation-regulated chemokine (TARC)
		CCL19	MIP3-β, macrophage inflammatory protein 3-beta
		CCL20	MIP3-α, macrophage inflammatory protein 3-alpha
		CCL22	MDC, macrophage-derived chemokine
		CCL23	Myeloid progenitor inhibitory factor 1
		CCL28	Cytokine induced neutrophil chemoattractant
	CXC chemokines:		
		CXCL1	KC, Gro-1
		CXCL2	MIP2-alpha, Gro-2
		CXCL3	MIP2-beta, Gro-3
		CXCL5	Neutrophil activating peptide ENA-78
		CXCL6	Granulocyte chemotactic protein 2
		CXCL10	Interferon-γ-inducible protein, IP-10
		CXCL11*	IFN-γ-inducible protein, IP-9
	Other:	CX3CL1	Fractalkine

Type		NF-κb target	Alternate names
Cell adhesion molecules:			
		CD44	Hyaluronic acid receptor
		CD56	Neural cell adhesion molecule
		CD62E	E-selectin, endolthelial cell leukocyte adhesion molecule
		CD209	DC-SIGN
		ICAM-1	intercellular adhesion molecule (ICAM) 1
		fibronectin	
	Other:		
		E-selectin	CD62E, endothelial-leukocyte adhesion molecule
		MadCAM-1	mucosal addressin cell adhesion molecule
		Tenascin C	Cytotactin
		VCAM 1	vascular cell adhesion molecule
Immunoregulatory molecules:			
	Chemokine receptors:		
		CCR5	CD195, (receptor for CCL3-5)
		CCR7	CD197 orphan receptor
		CXCR1	CD128, CD181, IL-8RA
		CXCR2	CD182, IL-8RB
	Complement:		
		complement factor B	CFB
		complement component 3	C3
		complement receptor 2	CR2, CD21
	Toll Receptors:		
		TLR2	Toll-like receptor 2
		TLR4	Toll-like receptor 4
		TLR9	Toll-like receptor 9
	NLR/Nod-like Receptors:		
		Nod2	Nucleotide-binding oligomerizaiton domain protein 2
	TNF Receptors:		
		CD120B	p75/80 high affinity TNFR
		CD137	TNFRSF9
		CD40	TNFRSF5

Type		NF-κb target	Alternate names
Immunoregulatory molecules (continued):			
	T-cell modulator:		
		IL-2Rα	IL-2 receptor α-chain
		CD3γ	T-cell receptor γ chain
		TCR-β	T-cell receptor β chain
	Antigen presentation:		
		β−2-microglobulin	B2M
		CD80	B7-1
		CD86	B7-2
		MHC class I	H-2 Kb
		MHC class I	HLA-B7
		tapasin	TAP-Binding protein
	Immunoglobulins:		
		Ig ε heavy chain	
		Ig κ light chain	
		IgG Cγ1	
		IgG γ1	
		IgG γ4	
		Invariant chain II	
		Polymeric Ig receptor	
		CD23	Fcε receptor II
	Other:		
		BRL-1	B-cell homing receptor
		CD48	B-cell activation marker
		LMP2	Large multifunctional protease 2, Proteasome-related gene 2
		TREM-1	Neutrophil/monocyte Ig receptor
Acute phase response proteins:			
		Angiotensinogen	
		β-defensin 2	
		C4b binding protein	
		complement factor B	
		complement factor C4	
		CRP	C-reactive protein
		LBP	lipopolysaccharide binding protein

Type		NF-κb target	Alternate names
Acute phase response proteins (continued):			
		PTX3	Pentraxin 3
		serum amyloid A proteins 1, 2	
		tissue factor 1	
		urokinase-type plasminogen activator	
Stress response genes:			
	Oxidation related:	CYP2C11	Cytochrome p450
		CYP2E	Cytochrome p450
		CYP7b	Cytochrome p450
		iNOS/NOS2	inducible nitric oxide synthase
		NAD(P)H quinone oxidoreductase	(DT-diaphorase)
		Mn SOD	manganese superoxide dismustase
		SOD1	Cu/Zn Superoxide dismutase
		SOD2	Superoxide dismutase
		COX-2	cyclooxygenase-2
		ferritin H chain	
	Other:	Angiotensin II	
		phospholipase A2	
		HSP-90	Heat shock protein 90
Apoptosis regulators:			
		ASC	Apoptosis-associated speck-like protein containing a CARD
		Bax	Pro-apoptotic Bcl-2 homolog
		Bfl 1/A1	Pro-survival BCL-2 homolog
		Bcl-2	B-cell leukemia 2
		Bcl-xL	Bcl2 related protein, long isoform
		Bim	Pro apoptotic Bcl2 homolog
		Casp11	caspase 11
		c-FLIP	Casp8 and FADD-like apoptosis regulator
		Fas	TNFR6, CD95
		FasL	Fas Ligand
		IAP	inhibitor of apoptosis
		TRAF-1	TNF Receptor-associated factor 1

Type		NF-κb target	Alternate names
Apoptosis regulators (continued):			
		TRAF-2	TNF Receptor-associated factor 6
		XIAP	X-linked inhibitor of apoptosis
Transcription/Growth Factors and their modulators:			
	Interferon regulatory factors:		
		IRF-1	
		IRF-2	
		IRF-4	
		IRF-7	
	Rel/NF-κB proteins:		
		p100/p52	NF-κB2
		p105/p50	NF-κB1
		c-Rel	
		RelB	
	IkB proteins:		
		IκBα	NF-κB1A
		IκBβ	NF-κB1B
		Bcl-3	B-cell lymphoma 3
	Growth Factors:		
		G-CSF	Granulocyte colony stimulating factor
		GM-CSF	Granulocyte Macrophage colony stimulating factor
		M-CSF	Macrophage colony stimulating factor
		PDGF	Platelet derived growth factor
	Other:		
		A20	Tumor necrosis factor-alpha-induced protein 3
		Blimp-1	B lymphocyte-induced maturation protein
		c-myb	
		JunB	
		Skp2	S-phase kinase-associated protein 2
		TP53	Tumor protein 53, p53

[a] Source: www.NF-KB.org, an invaluable resource curated by the laboratory of Dr. Thomas D. Gilmore, Boston University, Boston, MA.

* indicates there is a κB site in the gene promoter, but not yet shown to be controlled by NF-κB ; or the gene expression is associated with increased NF-κB but has not yet been shown to be a direct target.

Chapter 2
Francisella tularensis

The bacterium *Francisella tularensis* is the causative agent of human tularemia and is one of the most pathogenic bacteria known. Tularemia was first described in 1911 (53), as a plague-like illness affecting ground squirrels in Tulare County, California. The disease was found to be caused by a new bacterium, *Bacterium tularense,* named after the location of its discovery. The first verified human case of tularemia was reported in Ohio in 1914, although similar descriptions of infection resembling human tularemia date as far back as 1653 (54). Edward Francis, a pioneer in the etiology and epidemiology of this disease, showed that the deer fly could act as a vector of the disease and that blood from one infected animal could cause disease in another. He also summarized the clinical manifestations of the disease from a study of over 800 cases, which he coined tularemia (55). *Bacterium tularense,* was later renamed *Francisella tularensis*, in honor of Francis for his seminal contributions to the field (54-57).

Tularemia, also known as rabbit fever, is a naturally occurring disease. It affects a variety of mammals including small rodents and humans, as well as birds, reptiles and fish (58). This zoonotic disease is transmitted to humans through handling of infectious material, contact with arthropod vectors, ingestion of contaminated water and aerosolization. Clinically, tularemia presents itself initially with non-specific symptoms such as fever, chills, headache, malaise and myalgia (54). Specific symptoms can vary

from mild to life threatening, which depends on route of entry (Table 2), dosage and bacterial strain (Table 3). Experimental models in mice show that lethality varies by route of infection in the following order (in increasing severity): intradermal, subcutaneous, intraperitoneal and pneumonic (59).

Table 2: Manifestations of tularemia infections[a]

Type	Transmission	Symptoms	Comments
Oculoglandular	Direct inoculation of the eye	Conjunctivitis, swelling, light sensitivity, pus	
Oropharyngeal	Ingestion of contaminated food or water	Pharyngitis, mouth ulcers, tonsillitis, lymphadenopathy	
Ulceroglandular	Skin or mucous membrane contact	Skin ulceration, lymphedema,	~30% of patients who have ulceroglandular tularemia develop pneumonic tularemia
Pneumonic	Inhalation	High fever, malaise, chills, cough, chest pain, dyspnea, delirium.	High mortality
Typhoidal	No clinical signs of portal entry, no skin or lymph node involvement	Severe systemic symptoms including high fever, splenomegaly, hepatomegaly, pulse-temperature dissociation. Gastrointestinal and pulmonary symptoms may develop.	~80% of patients who have typhoidal tularemia develop pneumonic tularemia. High mortality

[a] Adapted from (54,60)

Francisella is a Gram-negative coccobacillus. Four subspecies of *Francisella tularensis* exist (Table 3). Originally, classification of the subspecies was based on geographical location and virulence in subcutaneously injected rabbits. However, further classification was needed since overlapping geographical isolates were observed.

Differences in metabolic and growth profiles (for example, the ability to ferment specific types of sugars) were used to further characterize these subspecies. This has been termed biovar (or Jellison grouping) type A or type B.

Pathogenicities in humans differ among the different subspecies; *Francisella tularensis* subspecies *tularensis* or the type A biovar is the most virulent strain and is of greatest concern to human health, while *Francisella tularensis* subspecies *holarctica*, or type B, is less pathogenic. Left untreated, infections derived from type A biovars exhibit 5-30% mortality, while type B biovars exhibit almost no mortality (58,60,61). *Francisella tularensis* subspecies *novicida* was once considered a separate species (*Francisella novicida)* based on biochemical results and its initial description which described a lack of a capsule found in other subspecies (60). However, DNA analysis has shown it to be closely related to *F. tularensis* and, thus, many have reassociated it as *F. tularensis* subspecies *novicida,* although this terminology still lacks official standing (62). *Ft*. subspecies *novicida* is essentially non pathogenic to humans (58,60,61).

Since its early description in 1911, tularemia infections among laboratory workers were well noted. The high virulence of type A strains in humans requires specialized handling precautions and isolation equipment in the laboratory setting. Therefore, tissue culture and animal studies of infection with strains avirulent in humans have often been used to model and dissect human disease (Table 3). The live vaccine strain (LVS or *Ft.* LVS) was derived from the subspecies *holarctica* type B biovar by repeated passage on agar and in animals. *Ft.* LVS infection in mice approximates human and non-human primate diseases in terms of disease progression and tissue responses (63,64). In addition, *Francisella* subspecies *novicida* is also widely used as an investigational tool.

While this strain is considered avirulent in immunocompetent humans, it has increased virulence in mice over *Ft*. LVS based on lethal inhalation doses (see (65). The fully virulent type A strain, Schu S4, is also used to model disease in animals, but as a virulent human pathogen, it requires special biohazard safety applications. As with any model organism, some caveats must be taken into consideration in interpreting results. For example, in a mouse lung model of infection with the type A virulent strain, Schu S4, differences are seen in cell survival within inflammatory foci of the liver and this results in a loss of containment of *Ft.* antigens to these foci as compared to *Ft*. LVS infected mice (64). Moreover, some differences among cytokine responses between LVS-infected human and mouse-derived macrophages have been noted as well. For example, live *Ft*. LVS produces a robust inflammatory response in human monocyte derived macrophages (hMDM) as compared to macrophages derived from murine bone marrow (BMDM). Secretion of IL-1β (Interleukin 1β), CXCL8/IL-8 (CXC motif ligand 8/Interleukin 8) and CCL2/MCP-1 (Monocyte chemotactic protein 1) are produced in hMDMs but not in BMDMs; Moreover, no CXCL8 homolog exists in mouse (66). Still, these models remain an important tool for investigational studies of this disease where human data is not available.

Table 3: *Francisella tularensis* subspecies[a]

Subspecies	Biovar [b]	Distribution	Estimated LD_{50} dose in humans (CFU, s.c.) [c]	Representative Lab Strain
tularensis	A	North America	$<10^1$	ShuS4
holarctica	B	Primarily in Europe and Asia. Some cases in North America	$<10^3$	LVS (Live vaccine strain, non virulent in humans)
mediaasiatica	Similar to type B, but considered unique	Locations in the post-Soviet Republics in Central Asia	Not determined	Not determined
novicida or *F. novicida* (as a separate species)	C [d]	Isolated from a water sample in Utah, Australia	$>10^3$	U112

[a] Information adapted from (58,60,61)
[b] Biovar (biovariant) based on biochemical, growth and metabolic properties
[c] LD_{50} (Lethal dose at 50% mortality); CFU (Colony forming units); s.c. (subcutaneous injection)
[d] Listed as a distinct biovar in (60)

After 2001, intense investigations surrounding *Francisella* biology reemerged after the Centers for Disease Control listed *Francisella tularensis* subspecies *tularensis* as a Category A agent for its potential use as a bioweapon. This classification is based on a number of factors: 1. How easily the disease is spread, 2. High death rates and have potential for major public health impact, 3. May cause public panic and social disruption, 4. May require special action for public health preparedness. (www.CDC.gov). In fact, it was reported that as few as 10 CFU (colony forming units) of bacteria can cause human

disease and the bacterium has great potential for aerosolization. Untreated, mortality rates can be as high as 30% and there are no approved vaccines currently in use. In fact, in the early 20th century both the United States and the former Soviet Union had developed weaponization programs for *Francisella*. (67)

Chapter 2.1:
Francisella Infection

Francisella tularensis is considered to primarily infect macrophages (68,69), but there are reports of infection in other immune cell types such as dendritic cells (70), monocytes (71) and neutrophils (72,73). Infection of non-immune cells such as hepatocytes (68) and alveolar epithelial cells (74) as well as an extracellular phase in murine plasma (75) have also been identified.

Phagocytosis of *Ft.* by macrophages begins with the formation of pseudopod loops (76) which envelop bacteria to form a phagosome. To avoid lysosomal destruction, *Francisella* utilizes a phagosome escape mechanism before phagosome-lysosome fusion. Studies using *Francisella tularensis* subspecies *novicida* showed that bacteria-containing phagosomes mature into late endosomes as noted by the acquisition of markers such as RABs (Ras-Associated Proteins) and LAMPs (Lysosome-Associated Membrane Proteins), but not lysosomal proteases such as Cathepsin-D (77,78). In addition, the release of surface materials, proposed to be capsular or envelope derived, from *Ft.* results in the formation of small vesicles near the phagosome. Formation of these vesicles is associated with phagosome membrane disruption and bacterial escape (79). Within 2-4 hours after infection, bacteria escape into the cytosol, the environmental niche where extensive replication occurs (reviewed in (78,80)).

Dissemination of *Francisella* results in colonization of the reticuloendothelial system in lung, liver and spleen. In the liver, infection results in the formation of inflammatory foci called granulomas. The granuloma is a compact structure of immune cells and it is thought that development of such structures helps contain infection, thereby protecting surrounding tissue. *Francisella*-induced granulomas generally consist of large numbers of epithelioid macrophages as well as dendritic cells, lymphocytes and some neutrophils (68,81,82). In addition, a large influx of granuloma-associated myeloid derived suppressor cells (MDSC) has been reported (82). MDSC are a population of immature myeloid cells which can differentiate into macrophages, dendritic cells or neutrophils (83). Yet, other cell types outside the granuloma may also contribute to host responses. For example, although natural killer (NK) cells are not generally associated with granuloma structures (82), they are also considered important in the liver's immune response to *Ft*. infection. NK cells are best known for production of cytotoxic mediators such as granzyme-B and perforin to induce cell killing. In addition, NK cells are also a major subset of IFN-γ producing cells in the liver after *Ft*. infection (81).

Chapter 2.2: Virulence factors and host interaction

Virulence factors are molecular components or evolved mechanisms that allow a pathogen to establish a niche within its host and cause disease. Some common bacterial virulence factors include production of molecules involved in adherence, colonization, tissue invasion, or development of mechanisms to evade host immune reactions. Although characterization of *Francisella* infections and its transmission to humans, other hosts and reservoirs spans nearly a century, there is a paucity of information regarding its

virulence factors. A new era in this field has emerged over the last several years, largely due to sequencing efforts and the development of genetic tools necessary to manipulate the genome of this fastidious organism. Several genomic screens have been designed to identify potential virulence factors. Cumulatively, these screens identified over 365 *Ft.* genes that were upregulated after either *in vitro* or *in vivo* infection, including many with unknown functions (reviewed in (84)). Below is a discussion of some of the better-characterized virulence factors.

CAPSULE:

The outer surface of most *Francisella tularensis* subspecies contains a thin capsule, although it remains poorly defined. In general, capsules are considered an important protective mechanism for bacteria to survive in hostile environments. Consisting of caspsular polysaccharides, outer membrane proteins and LPS, capsules can help bacteria mask bacterial antigens, evade phagocytosis or use it as a decoy to activate cell receptors to gain entry into intraepithelial junctions. Different bacterial capsular components have also been reported to block host complement activation and killing (reviewed in (85)).

The complement system is a network of circulating inactive plasma proteases that, when activated, are cleaved to release mature proteases and anaphylatoxins. This cascade results in the eventual formation and deposition of several complement proteins on the bacterial surface. Importantly, complement C3, an opsonizing protein that increases phagocytosis by host cells, and the C5b-9 proteins, which form osmotic pores termed the

membrane attack complex (MAC), are deposited on the microbial surface (reviewed in (86,87)).

Early work using a capsule deficient mutant of *Ft.* LVS, (CAP-), highlighted the importance of the capsule for virulence. The capsule protected wild type parental strains from serum-mediated opsonization over CAP- strains. Opsonization with serum IgG (immunoglobulin G) or complement in normal human serum (88,89) targets non-capsulated bacteria for destruction by leukocytes. CAP- mutants also displayed decreased virulence *in vivo* (88). In addition, genetic screens identified *cap A,B* and *C* genes in *Ft*. LVS, *novicida* U112 and *tularensis* Schu S4 species (84). These genes are considered putative capsular genes, as they are homologous to the *Bacillus anthracis* capsule biosynthesis genes. Moreover, a recent finding shows that infection with a mutant containing a targeted deletion in the *capB* gene is more attenuated in mice than its parental *Ft.* LVS strain by intradermal and intranasal route of infection (90), which agrees with the CAP- results. In terms of complement activation, C3 complement fragments do bind the bacterial surface, however, the MAC complex is unable to form and bacteria escape complement-mediated cell lysis (91), further indicating that the capsule is an important virulence factor for *Ft*. survival.

LPS:

The outer membrane of *Francisella* also contains LPS; however, due to an unusual O-linked polysaccharide structure, it is not well recognized by host TLR4 (Toll-like receptor 4), an LPS receptor for recognition of O-linked mannans. A comparison of

Ft. LPS to *E. coli* LPS shows that *Ft.* LPS is only very weakly activating and does not confer resistance to subsequent infection, indicating it has very low endotoxicity (92-94).

FRANCISELLA PATHOGENICITY ISLAND:

Pathogenicity islands generally consist of large genomic regions (up to 200 kilobases of DNA) that contain virulence genes such as: adhesins, which allow microbes to attach to specific receptors; secretion systems, which are important for secretion of virulence factors or for entry into a host cell; invasins and modulins, which help bacterial entry or to modulate specific host signaling pathways, among others (reviewed in (95)). Sequencing of *Ft*. genomes led to the discovery of a 33kb (kilobase) pathogenicity island (FPI, *Francisella* pathogenicity island) (96). Type A and type B *Francisella tularensis* strains contain two copies of the FPI, whereas the *novicida* U112 strain contains just one (84). For this reason, much of the single-gene mutational analysis to find associated gene function has been carried out in the U112 strain. However, most FPI encoded genes are well conserved among *Francisella* species (see (97)).

The pathogenicity island of *Fransicella tularensis* subspecies *novicida* contains 19 genes (96,97). Many of the *Fransicella* open reading frames (ORFs) remain uncharacterized or lack identity in other species of bacteria and, therefore, associated proteins are generally categorized as “hypothetical” and/or “orphans”. Prior to mapping of the FPI, some genes, such as those contained within an operon termed *IglABCD* (Intracellular growth locus A-D), were already understood to be virulence-related based on their defects in intracellular growth (98). Table 4 lists some of the genes located on the FPI, as well as their putative functions delineated from mutational analysis studies.

Table 4: Selected *Francisella* Pathogenicity Island genes

FPI gene	Proposed function by mutational analysis	References
IglA	Intracellular growth.	(99,100)
IglB	Intracellular growth. Stability of IglA.	(99-101)
IglC	Intracellular growth. Phagosome escape, prevention of phagosome-lysosome fusion.	(99,100) Protein identified in (102-104).
IglD	Intracellular growth. Not involved in phagosome escape.	(99,100,105)
PdPA	Intracellular growth. Phagosome escape. Cannot induce macrophage apoptosis.	(99,100,106)
PdPB	Phagocytic uptake.	(107) Protein identified in (103)
PdPC	Intracellular growth and virulence	(108) Protein identified in (104)
PdPD	May direct the cellular distribution of IglA and IglC proteins. Association with type VI secretion system. May not be required for intracellular growth Not present in subspecies *holarctica*[a]	(96,109)

[a] (108)

FPI TRANSCRIPTIONAL REGULATION:

Several FPI transcriptional regulators have been found. Two are RNA polymerase interacting proteins MglA (macrophage growth locus A) (100) and SspA (homolog of the *E. coli* Stringent starvation protein A) (110). FevR (Francisella effector of virulence regulation), which has limited homology to the helix-turn-helix, DNA

binding transcription factor family MerR (Mercury resistance), has a role in regulating the *iglABCD* operon (111). MigR (macrophage intracellular growth regulator), is a regulator of *fevR* expression, thus, it indirectly regulates the *iglABCD* operon (112). PmrA (Polymyxin resistance A) is part of an orphan two-component regulatory system that induces transcription of 65 genes in response to certain environmental cues (113). Finally, Hfq (homolog of Host Factor of the Qβ RNA phage in *E. coli*) acts mainly as a negative regulator of FPI transcription (114). Mutations have been made in each of these genes that highlighted their roles in FPI transcription as well as virulence.

The best-studied regulator of the FPI is MglA. A spontaneous mutant of *Ft.* subspecies *novicida* lacking acid phosphatase activity was isolated (115). This mutant failed to replicate in macrophages, indicating the gene(s) responsible for the mutation was vital to virulence. A complementation assay led to the discovery of an operon that contained two genes, *mglA* and *mglB* (macrophage growth locus A and B). Sequencing analysis of MglA and MglB proteins showed some similarities to *E. coli* RNA polymerase holoenzyme-associated proteins, SspA and SspB*,* indicating a potential role in transcription. In agreement with this observation, protein analysis revealed a loss of expression of several other FPI proteins ((115) and reviewed in (108)). In fact, MglA was found to regulate transcription of over 100 genes, and, importantly, it is required for the expression of known FPI virulence genes *IglA-D, PdpA* and *PdpD,* as well as for several hypothetical genes of unknown function also located on the FPI (99,100,116). A full characterization of the *mglB* mutant has not been carried out thus far, although it has been reported to be required for the expression of PdpA, PdpB and IglB (117,118).

The *mglA* mutant is highly attenuated for virulence in mice (100). Electron microscopy images revealed this mutant is defective in phagosomal-escape (106). The inability of *Ft.* mutants to escape the phagosome blocks not only its cytosolic replication, but restricts interactions with host proteins as well, for example, *Ft.* subspecies *novicida* with a mutation in *mglA* or *pdpA* do not activate the inflammasome (106).

The inflammasome is an induced complex of proteins that leads to activation of caspase-1 (119). Caspase-1 mediates processing of pro-proteins pro IL-1β (interleukin-1β) and pro IL-18 (interleukin 18) cytokines into their active forms. Activated, IL-1β and IL-18 are potent cytokines for cellular recruitment and coordination of proinflammatory and cellular responses (120). In addition, prolonged caspase-1 activation can lead to cell death (121).

Inflammasomes generally consist of a sensor protein (Nalps (Nacht domain, leucine-rich repeat and pyd-containing proteins); IPAF (ICE Protease Activating factor); NAIP (Neuronal apoptosis inhibitory protein); AIM2 (Absent in Melanoma)), a scaffolding adaptor ASC, (Apoptosis-associated speck-like protein containing a caspase-associated recruitment domain)) and Caspase-1/ICE (interleukin converting enzyme) (reviewed in (122,123)).

The *Ft*. induced inflammasome contains the AIM2 (124) as a cytosolic DNA sensor, the ASC adaptor protein, and caspase-1 (106). Mice with deletions in either ASC or Casp-1 do not produce IL-1β and exhibit little cell death, underlining their importance in host control.

Signaling crosstalk between the inflammasome and NF-κB pathways occurs at the level of NF-κB dependent transcriptional control of pro-IL-1β. Recently it was shown

that although loss of IKKβ in macrophages decreases the available pro-IL-1β transcripts available for protein synthesis, it paradoxically, increases processing of pro-IL-1β producing more secreted IL-1β than WT mice. Furthermore, blocking with IL-1R antagonist protected IKKβ deleted mice from IL-1β mediated septic shock and death (125). Whether the survival requirements for macrophage or neutrophil IKKβ in *Ft.* LVS infection model depends on canonical NF-κB signaling output, inflammasome integration and/or IL-1β processing control will require future investigation.

SECRETORY PATHWAYS

Gram-negative bacteria assemble protein machinery in the form of pores in order to transport secretions across their inner and outer membranes. These secretion systems are dependent on ATP hydrolysis in order to move solutes across membranes and/or regulate the aperture of the pore itself. Six types of bacterial secretion systems have been described Type 1-VI or T1SS-T6SS (reviewed in (126)).

The search for a functional secretion system in *Francisella* began with a bioinformatics search to look for homologous secretion system related genes.   Two candidate genes, *tolC* and *ftlC* showed some homology to the *E. coli tolC* gene (127). TolC is a trimeric protein that embeds in the outer membrane of Gram-negative bacteria and is reported to function in Type 1 Secretion Systems (T1SS) as well as multi-drug efflux pumps. Type 1 secretion systems are structurally simple, composed of just 3 proteins, an outer membrane pore forming protein (such as *tolC* or *ftlC*), a membrane fusion or adaptor-like protein and an inner membrane ATP pump. Typical secretions

from bacterial T1SS include: hemolysin A, metalloproteases, lipases, hemophores and adhesion factors (128)

Mutational analysis of these genes in *Ft.* subspecies *novicida* revealed, in a multidrug sensitivity assay, that both genes likely function as a drug efflux pump (127). TolC and FtlC are not required for bacterial replication or dissemination to lung, liver or spleen, indicating that they are not involved to cell entry or phagosomal escape. Bacterial organ burdens are, however, lower in *tolC* mutants and are attenuated in infected mice as compared to WT or *ftlC* mutants (129). Other constituents of the T1SS and/or potential secretory proteins from these pumps have not yet been described, but the *in vivo* attenuation of a *tolC* mutation, indicates a role other than that of a multi-drug efflux pump. Since these mutants are not defective in replication or dissemination, it may be that the putative TISS is involved in protection from immune surveillance at an intermediate or later time point during the course of infection and that the lower levels of organ burden are due to decreased bacterial survival.

In 2006, a novel secretion system termed the Type VI Secretion System (T6SS) was discovered in *Vibrio cholerae* and *Pseudomonas aeruginosa* (reviewed in (130)). As a relatively new discovery, less is known about them. This secretion system was initially defined by just a minimal set of characteristics, such as: the presence of an AAA+ family Clp-like ATPase (AAA+ = ATPases associated with various cellular activities; Clp = caseinolytic peptidase), the presence of two genes, *icmF* (intracellular multiplication F) and *dotU* (defect in organelle trafficking U) as well as secretion proteins of the Vgr (Valine-Glycine Repeat) and Hcp (haemolysin co regulated protein) families (130).

Several groups used a bioinformatics approach to see if analogous T6SS encoding regions of *V. cholerae* and *P. aeruginosa* exist in FPI gene clusters. These studies identified *iglA, iglB* (101), *pdpB* (pathogenicity determinant protein B, an *icmF*-like gene), *vgrG, clpV, dotU* and *hcp* (108) genes as having some, albeit sometimes limited, homology to genes encoding a T6SS. In addition, two proteins, VgrG and IglI, are secreted into the cytosol of J774 macrophage-like cells after *Ft.* subspecies *novicida* infection (108). IglI does not have homology to any known T6SS orthologous genes, and may represent a unique *Ft*. specific virulence factor.

Recently, a large scale *in silico* study of over 500 bacterial genomes examined multiple sets of conserved orthologous genes (COGs) in order to more accurately define T6SS conserved genetic structure, evolution and organization within the genome (131). This analysis identified 176 encoding regions from 92 different bacteria. Phylogenetic analysis revealed T6SSs are composed of 13 conserved core proteins (of which, 8 have been characterized) as well as a conserved set of accessory proteins. An important feature in T6SS is that the genes encoding these proteins are also considerably organized within the genome. In this screen, only 3 *Ft*. FPI genes, *iglA, iglB* and *pigF* (pathogenicity island gene F) show any significant homology to a T6SS and raised questions as to whether the *Francisella* FPI actually encodes a T6SS, or perhaps these genes may represent a modified T4SS (Type IV secretion system) (131).

This discrepancy is likely due to the limited homology between the putative T6SS genes described in Barker *et al.* (108) and T6SS genes of other bacterial systems and may require a better understanding of uncharacterized FPI and non FPI genes. Through mutational analysis, classification of several *Ft* virulent genes has been carried

out and their effects in an *in vitro* or *in vivo* host environment has been systematically described; However, the actual function of many of these genes are still unknown (hence, generic names assigned to them, such as: intramacrophage growth locus, pathogenicity island gene, etc.). It is possible that these “orphan” genes may reveal other secretion related genes and possibly that *Francisella spp*. lack the organized genetic structure of T6SS observed in other bacterial genomes.

Chapter 3
Host immune reactions

Immune responses toward foreign antigens can be broadly defined into two broad subclasses: innate and adaptive. Innate immunity is considered less specific and includes host protection mechanisms such as physical barriers like skin and mucosa, production of chemical mediators such as complement and other anti-microbial substances as well as cellular effector functions such as phagocytosis. Adaptive immunity is mediated through development of specific antibodies in response to foreign antigen, the development and selection of specific cellular subtypes, which help shape the immune reaction and immunologic memory. In addition, innate immunity occurs immediately or within hours of contact while adaptive immunity is delayed until the production and selection of specific antibodies occurs (132).

Several cell types are involved in mounting immune reactions towards *Francisella* infection. This chapter will focus on some of the signaling pathways leading to immune activation in response to *Francisella* infection as well as the cell types and their corresponding functions surrounding my research.

Chapter 3.1
Immune signaling

Host immune recognition of bacteria and other microbes occurs through specialized receptors called Pattern Recognition Receptors (PRRs). These receptors recognize a set of conserved molecular patterns found in microbes termed PAMPs (Pathogen Associated Molecular Patterns). The PRRs include the TLR (Toll-like receptors), RLRs (RIG-I-like receptors, RIG-I = Retinoic-acid-inducible protein-I), NLRs (NOD-like receptors) and CLRs (C-type lectin receptors). Of these, the TLRs and NLRs are involved in recognition of bacterial PAMPs such as lipoproteins, flagellin, nucleic acids and peptidoglycans (Reviewed in (133)).

There are eleven known TLRs and each differs in recognition of and affinity for different microbial substrates. For example, TLR2 is involved in recognition of lipoproteins, some types of lipopolysaccharides (LPS, e.g. *Leptospira, Porphyromonas gingivalis*) and zymosan (a protein-carbohydrate moiety from yeast cell wall) while TLR4 is involved in recognition of some Gram-negative LPS (e.g. *Escherichia coli*), taxol (found in plants), some host derived products such as HSP60 (Heat shock protein 60) and fibronectin. TLR5 recognizes flagellin, and TLR9 is associated with recognition of unmethylated CpG DNA (reviewed in (56,134)).

Several groups reported on the involvement of TLR2 *in vitro* and *in vivo* in response to *Ft*. LVS (135-140). TLR2 forms heterodimers with either TLR6 or TLR1 and recognizes di- or tri-acylated lipoproteins respectively. Dimerization takes place through protein-protein interactions between the cytoplasmic TIR (Toll/Interleukin receptor) domains of each TLR (133). *Ft.* LVS infected TLR2$^{-/-}$ peritoneal elicited

macrophages show diminished TNF-α (tumor necrosis factor α), IL-1β (interleukin 1β), KC (mouse homolog of CXCL1), IL-12p40 (interleukin-12 subunit p40), RANTES (regulated upon activation, normally T-expressed and presumably secreted), IFN-γ (interferon-γ), IFN-β1 (interferon β1) and iNOS (inducible nitric oxide synthase 2) cytokine/chemokine expression over WT animals (138). *In vivo*, TLR2$^{-/-}$ mice show increased mortality, increased bacterial burdens in lung, liver and spleen as well as decreased TNF-α and IL-6 (interleukin 6), but increased IFN-γ and MCP-1 (monocyte chemotactic protein 1) in lung homogenates (136).

In addition to TLR dimerization, the TIR domain is also important for the recruitment of TIR-domain containing signaling adaptors. Mal/TIRAP (MyD88 adaptor-like/TIR domain-containing adaptor protein) and MyD88 (Myeloid differentiation protein 88) are two such TIR containing adaptors recruited to TLR2 upon receptor engagement (133).

The Mal adaptor serves as a bridging protein for Myd88 in TLR2 and TLR4 signaling. The TLR/Mal/MyD88 complex leads to NF-κB activation and expression of inflammatory mediators such as TNF-α, IL-6 and pro IL-1β (141). Mal itself requires caspase-1 dependent C-terminal cleavage in order to potentiate NF-κB activation (142). However, Mal can also function independently of MyD88. In response to diacylated lipopeptide, Mal activates the p85α regulatory subunit of PI3K (phosphoinositide-3-kinase), and is essential for TLR2/6 but not TLR2/1 dependent PI3K activation. Activation of PI3K can lead to downstream activation of Akt (V-Akt murine thymoma viral oncogene homolog) (143), as well as modulate other pathways and cellular

functions depending on cell type (144). Furthermore, in the presence of MyD88, PI3K activation can enhance transactivation of the p65 NF-κB subunit (143).

MyD88 is a universal TLR adaptor and, as such, is required for signaling in all TLRs except for TLR3, where it acts a negative regulator. In addition, MyD88 functions in IL-1β and IL-18 receptor (145) signaling as well. The function of MyD88 is to recruit downstream adaptors such as IRAK (IL-1 receptor associated kinase) to activate NF-κB (141).

In vivo, TLR2 is required for host immune activation against *Francisella* (136,140). In a lung infection model, TLR2$^{-/-}$ mice succumb faster and more often to lethal infection. In addition, these mice have an impaired ability to control bacterial growth or clear infection from lung, liver and spleen. Furthermore, bacterial replication in the lung fails to resolve. This was also correlated to a loss of production of the inflammatory cytokines TNF-α and IL-6 (interleukin 6) (136). *In vitro*, peritoneal elicited macrophages (PEMS) (138) and dendritic cells (DCs) (135,139) show similar requirements for TLR2 to control infection.

In order to better determine which TLR2 heterodimer was involved in *Francisella* infection, one study used dendritic cells (DCs) from TLR deficient to evaluate the contributions of several TLRs. DC's derived from TLR4 or TLR1 deficient mice were able to induce TNF-α (tumor necrosis factor alpha), a well-known TLR inducible NF-κB target gene, in response to infection. Contrary to this, TLR2 and TLR6 deficient mice were cannot induce a TNF-α response, indicating that the functional heterodimer is TLR2/6 (135).

In another study, Li and coworkers (137) cotransfected several different TLR components and an NF-κB luciferase reporter into HeLa cells (an immortalized, epithelial-derived, cervical carcinoma cell line) to reconstruct and define functional TLR dimer(s) activated by *Ft*. LVS. Li's results suggest that, indeed, TLR2/6 is activated by *Ft*. infection, but contrary to the DC results shown by Katz *et al.* (135), TLR2/1 is also activated (137). Although the constructs in this experiment were entirely artificial (involving cytoplasmic and extracellular TLR domain swapping and subsequent expression in HeLa cells), the authors did test them rigorously for response to other di- and tri- acylated lipoproteins specific to either TLR2/1 or TLR2/6. Moreover, this same group recently identified two *Francisella* lipoproteins, TUL4/LpnA and FTT1103, which activate TLR2/1 using the same domain-swap experimental model (146). Yet, an LpnA deletion mutant in the *Ft*. LVS strain does not affect bacterial virulence or dissemination to lung liver or spleen in mice (147). On the other hand, mutation of the lipoprotein FTT1103 resulted in avirulence, as it is required for intracellular bacterial growth, replication and dissemination *in vitro* and *in vivo* (148).

The TIR domain containing proteins Mal and MyD88 were also studied in the context of *Ft.* infection. Peritoneal elicited macrophages from TLR2$^{-/-}$, Mal$^{-/-}$ or Myd88$^{-/-}$ mice all exhibit blunted TNF-α and IL-1β mRNA production in response to *Ft*. LVS infection (149). Interestingly, the molecular requirements for signaling are different when bacteria are retained within the phagosome. In this case, WT and Mal$^{-/-}$ macrophages exhibit increased and prolonged TNF-α and IL-1β mRNA production when infected with the phagosomal escape mutant, *Ft*. LVS Δ*iglC*. Conversely, MyD88 is absolutely required as no cytokine response is elicited after infection under the same

conditions. This suggests that activation of these cytokines can take place from within the phagosome in a Mal, but not MyD88 independent manner (149).

MyD88 knockout mice are extremely sensitive to *Ft*. LVS infection. Survival rates are reduced even when infected at doses well below those considered to be sublethal in WT mice. MyD88 mice also exhibit increased organ burdens in lung, liver and spleen. IL-12 (interleukin 12) and IFN-γ (interferon γ) are two cytokines involved in establishment of an inflammatory T_H1 response (discussed later in this chapter). Loss of MyD88 results in suppression of IL-12 and ablation of IFN-γ responses in infected mice (150).

In addition to the fact that the Toll receptor family leads to NF-κB activation (reviewed in (151)) other indications that *Ft*. affects the NF-κB pathway came about in two studies which showed that *Ft*. can prevent degradation of the NF-κB inhibitor IκBα and prevent the induction of TNF-α and IL-1 (152,153). Surprisingly, bacteria were also able to suppress cytokine production from cells stimulated with *E coli* LPS, indicating this may be an active inhibition by *Ft*. possibly upstream of IκBα. *Francisella* phagosome escape is dependent on a 23KDa protein denoted IglC. These mutant bacteria were not only unable to inhibit LPS mediated TNF-α or IL-1 production, they in fact enhanced it (153). This means that bacterial interruption of NF-κB activation takes place in the cytosol and leads to the intriguing possibility that bacteria may directly interact with NF-κB signaling components.

Chapter 3.2
Cellular Components
of *Francisella* mediated immunity

Macrophages are a heterogenous set of immune effector cells involved in phagocytosis, cytokine production, wound repair and antigen presentation as well as production of signals to coordinate the recruitment of leukocytes. Tissue macrophages are found throughout the body such as: Kupffer cells (KCs) in liver, alveolar and interstitial macrophages in lung, osteoclasts in bone and microglia in neuronal tissue. They function not only as sentinels of the immune system but also in tissue homeostasis by removing dead cells and debris from the local environment as well as recycling of red blood cells. In addition to resident tissue macrophages, infiltrating macrophages are derived from circulating monocytes, which are activated by chemical signals from injured tissue and extravasate to the sites of injury where they then terminally differentiate.

Macrophages are activated by cytokines and other signals, and depending on the stimulation, macrophages mature with specialized functions. Recently a system of classification has been adopted to describe macrophage activation. This classification is fashioned after that which was adopted for T_{HELPER} cells (T_H1 and T_H2 classification system) and is based on cytokine activation and production profiles (summarized in Table 5 and reviewed in (154)).

Table 5: Macrophage polarization

Macrophage	Activating Stimuli	Effector function	Cytokines/Enzymes produced
M1 (classical)	IFN-γ, IFN-γ + LPS, LPS, microbes.	Proinflammatory, phagocytosis, Development of T_H1 responses	Increased IL-12, iNOS, Low IL-10
M2a (alternatively activated)	IL-4, IL-13	Anti-inflammatory, suppress T_H1 responses	Increased IL-10, Increased MHCII, Arg-1, Fizz1, mannose receptor
M2b	LPS/IL-1β + FcγR stimuli	Development of T_H2 adaptive responses, antibody production	High IL-10 (higher than M2a), TNF-α, IL-1β, IL-6, Low IL-12
M2c	IL-10, TGF-β, glucocorticoids	Deactivated macrophage, increased scavenging activity	CCL18

In addition to the plasticity macrophages exhibit when exposed to different cytokine milieus, they also differ at the tissue level. Comparisons between KCs and alveolar macrophages show that KCs are highly specialized in endotoxin clearance from the gut; KCs also exhibit greater phagocytic ability and have been reported to maintain a constitutive state of low-level activation. Alternatively, alveolar macrophages are activated upon exposure to inhaled contaminants and react with increased cytotoxic mediators including increased ROS (Reactive Oxygen Species) and RNI (Reactive Nitrogen Intermediates) (reviewed in (155)). Organ tolerance to inflammation or infection as well as tissue specific needs to maintain organ homeostasis and function may be the most likely reason for this.

Chapter 3.3
Neutrophils

Neutrophils, also known as polymorphonuclear cells (PMNs), are myeloid-derived phagocytic cells important as a first line of defense towards bacterial infection. They are the most numerous circulating cell-type and are short-lived (6-12 hours in vivo) due to a constitutive apoptotic program. It is thought that this short lifespan is be due to their extreme cytotoxic potential and therefore, must be tightly regulated (156,157). Recruitment of neutrophils to sites of localized infection or inflammation is a rapid process that occurs within minutes to hours. The process begins when circulating neutrophils marginate to the peripheral endothelial surface and interactions occur between the two cell types. Neutrophils constitutively express the cell adhesion molecule L-selectin while endothelial cells express E-selectin and P-selectin only after activation resulting from injury, inflammation or infection. Interaction of these molecules mediates a loose attachment and allows the neutrophil to "roll" along the vessel surface. A further tight adherence is required for the cell to stick to the surface and prepare for transmigration into the tissue parenchyma. This is mediated by upregulation of other cell adhesion molecules such as the integrin-containing receptors CD11a/CD18 and CD11b/CD18 on the neutrophil and ICAM or VCAM on endothelial cells. Once an attachment is established, transmigration occurs as neutrophils move toward a gradient of chemokines, such as the ELR^+ CXC chemokines (chemokines that have a glutamic acid-lysine-arginine motif adjacent to a CXC motif are considered neutrophil specific chemokines) released by cells at the site of infection or injury (158).

Neutrophils also undergo an activation process that is mediated by microbial products or host-derived cytokines. Activation has multiple effects on neutrophils. First,

it suppresses apoptosis and extends neutrophil lifespan up to 48 hours. This is important in that the neutrophil will have sufficient time to perform its functions at the site of damage. In addition, activation promotes degranulation (the ordered release and secretion of granules which contain migration-associated proteases and antimicrobial substances) and secretion of proinflammatory cytokines and/or chemokines important in containment and clearance of bacteria, parasites and viruses. Furthermore, activation increases neutrophil effector potential including the generation of reactive oxygen species (ROS), termed "respiratory burst", and reactive nitrogen intermediates (RNI) (157). ROS and RNI generate the reactive radicals and intermediates of microbicidal mediators such as H_2O_2 (hydrogen peroxide), peroxynitrates or nitric acid. The damaging effects of ROS and RNI include DNA damage as well as oxidation or nitrosylation of lipids and proteins (see Figure 3 and (159)).

One way in which neutrophils produce reactive oxygen involves assembly of the NADPH oxidase complex at the plasma or phagosomal membrane. This complex is composed of phox (phagocyte oxidase) proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the transmembrane complex $cytochrome_{b558}$ (composed of $gp91^{phox}$ and $p22^{phox}$ proteins). These proteins are spatially separated in quiescent cells and, upon activation, phosphorylation of $p47^{phox}$ unmasks binding domains, which nucleate the assembly of phox proteins and further association with $cytochrome_{b558}$ occurs in a RAC-dependent (Rho family small GTP-binding protein) manner (reviewed in (156)). Assembly of the NADPH oxidase in the phagosome leads to oxidation of bacterial proteins and lipids. In addition to this microbicidal effect, reactive oxygen can also enter the cytosol, affecting

the redox state of the cell and activate secondary signaling molecules to regulate cell function (160).

Reactive nitrogen is generated from the metabolism of L-arginine to L-citrulline by nitric oxide synthase (NOS). Two isoforms of NOS are expressed constitutively while iNOS/NOS2 (inducible nitric oxide synthase) is induced by inflammatory stimuli and cytokines. As with ROI, RNI are also have a role in modulation of immune function as well, as they can act on G-proteins, kinases, caspases, metalloproteases, transcription factors and more (reviewed in (161)). In addition to the generation of ROS, RNI and microbicidal products of these pathways, the intermediate radicals $O2^{-\bullet}$ and $NO^{\bullet}$ also interact leading to generation of reactive hydroxyl ($OH^{\bullet}$) or nitrogen dioxide radical ($NO2^{\bullet}$) (161).

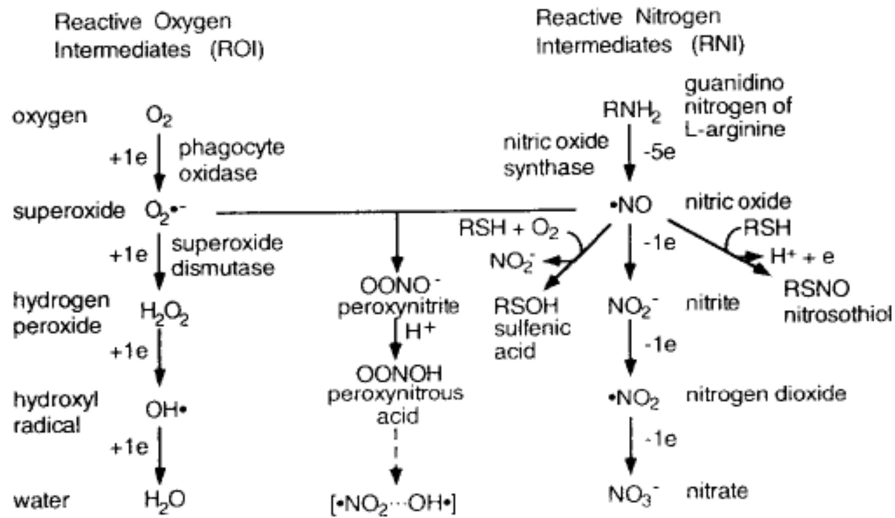
There is evidence for requirement of neutrophil function *in vivo*. Neutrophils accumulate at *Ft*. foci in the liver within 24 hours of infection and are critical for bacterial control and host survival during *Ft*. infection (68,72). The RB6-8C5 monoclonal antibody (also known as Gr-1/Ly6c Ly6g) depletes neutrophils and eosinophils in mice. *Ft*. LVS infected, RB6-8C5 treated mice rapidly succumb to intradermal infection at much lower doses than untreated mice. This correlates to increased bacterial cell counts in lung, liver, spleen and skin. Moreover, in the liver, an excess of dissolving hepatocytes indicates a potential role for neutrophils in clearance of infected hepatocytes (72).

A lung infection model using Francisella subspecies novicida showed an atypical negative neutrophil response within the first 6 hours after intranasal instillation. This

delay was also associated with a reduced expression neutrophil specific chemokines including ELR+ CXC (162). In fact, neutrophil migration did not occur until 24 hours post infection.

Studies in human neutrophils with Type A or Type B virulent *Francisella* strains (and also the attenuated strain *Ft.* LVS, which is avirulent in humans) show that the oxidative respiratory burst is interrupted. This occurs at two separate points of NADPH oxidase assembly. First, there is decreased PKC (protein kinase-C) mediated phosphorylation of $p47^{phox}$, an event important in the initiation steps of NAPDH oxidase assembly. The second disruption of NADPH oxidase formation by Francisella involves a defect in the recruitment of $gp91^{phox}$ and $p22^{phox}$ to phagosomal membranes (73,163).

FIGURE 3:



ROI and RNI production in mammalian cells via phox and NOS: parallel but connecting paths.

Nitroxyl anion (NO^-), a one-electron reduction product of nitric oxide (·NO), is unlikely to arise from ·NO under physiologic conditions, but is considered by some investigators to be primary and more toxic product of NOS. Reaction of RNI with cysteine sulfhydryls can lead either to S-nitrosylation or to oxidation to the sulfenic acid, as well as to disulfide bond formation (not shown), all of which are potentially reversible. Peroxynitrite anion ($OONO^-$) and peroxynitrous acid (OONOH) have distinct patterns of reactivity. OONOH sopontaneously decomposes via species resembling the reactive radicals, hydroxyl (OH^-) and/or nitrogen dioxide ($\cdot NO_2$). When L-Arginine is limiting, NOS can produce superoxide (O_2^-.) along with ·NO, favoring the formation of peroxynitrite. Copyright (2000) National Academy of Sciences, USA (161).

Chapter 3.4
NATURAL KILLER CELLS

Natural killer (NK) cells are a lymphoid derived cytolytic cell population. Originally they were described as “natural killers” because they could initiate an apoptotic program in tumor cells without prior activation (164). Studies in human NK cells show they are recruited to sites of inflammation by chemokines, many of which are NF-κB gene ouputs, including: IP-10/CXCL10 (interferon gamma inducible protein 10), MIP-1a/CCL3L1 (macrophage inflammatory protein 1a), RANTES, MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7 and MIP-1b/CCL4 (165). NK induced killing occurs on those cell whose missing self-expression peptides in the context of MHC class I are missing or downregulated (164). Killing involves cell-cell contact and the subsequent release of perforin and granzyme containing granules to induce apoptosis of the target cell (166). In addition to direct killing, NK cells produce inflammatory cytokines such as TNF-α and IFN-γ. TNF-α is a proinflammatory cytokine that activates NF-κB and directs the transcriptional response of genes involved in inflammation, cell cycle and/or apoptosis. Activated NK cells secrete IFN-γ and this has effects on several other cell types (167). Of special importance is its prominent role in directing classical macrophage activation by setting up the proinflammatory T_H1 response by $CD4^+$ T helper cells. . In addition, NK activation and IFN-γ further stimulate T cell subsets to produce IFN-γ as well (168).

IFN-γ’s effect on macrophages increases antigen presentation potential, lysosomal function and is required for activation of the microbicidal enzyme NOS2/iNOS (inducible

nitrous oxide synthase). IFN-γ also drives T_H1 proinflammatory pathways while suppressing T_H2 adaptive immune responses (reviewed in (169)). IFN-γ was found to be essential for initial control of infection, as loss significantly reduces the LD_{50} (lethal dose of bacteria required to kill ½ the population) (170). Furthermore, IFN-γ is considered to be essential in the control of granuloma formation during *Ft*. infection. Mice deficient in IFN-γ form fewer and smaller granuloma structures. Loss of these structures is correlated with the spread of *Ft*. antigen throughout the liver parenchyma. In this context, IFN-γ is also necessary for iNOS production and limiting necrosis as seen by increases in TUNEL+ (Terminal deoxynucleotidyl transferase dUTP nick end labeling, an assay for detecting fragmented DNA which occurs as a result of apoptosis) cells in knock out mice. (81)

Chapter 3.5
T cells

Lymphocytic T cell precursors originate in the bone marrow then home to the thymus where they mature. Maturation can be followed by the expression of two antigens, CD4 and CD8 (clusters of differentiation 4 and 8). Although several other T cell subsets exist, (γδ T cells, regulatory T cells, memory T cells), CD4 and CD8 cells define the two largest classes of T cells. During thymic development, T cells begin to express their T cell receptor (TCR). The TCR undergoes genetic rearrangement and this induces the expression of CD4 CD8 double positive T cells. These cells must then go through a process of clonal selection to yield cells that are capable of recognizing MHC (Major Histocompatiblity Complex) molecules with proper avidity. Cells that recognize MHC II mature into CD4 single positive T cells and those that recognize MHC I develop

into CD8 single positive T cells. Lymphocytes who pass this selection process are considered mature, but naive. These cells are then released into the circulation upon which they can encounter antigen (132,171).

CD4 T cells are classified as “Helper” (T_H) cells, named for their role in helping the synthesis of antibody production. In 1986, the helper cell subset was further subdivided into T_H1 and T_H2, as each set can be classified by different functional properties and expression patterns (172). A similar classification has also been made for CD8 T cells. These are classified as T_C1 and T_C2 cells (173). These cell types are summarized in Table 6.

CD4 T cells are well recognized for their interactions with professional antigen presenting cells (APCs), such as DCs and macrophages. When an APC has engulfed a pathogen, foreign proteins are cleaved into small peptides within the phagosome and then exported and presented on the cell surface bound to the MHC class II complex. The T cell receptor (TCR), along with the costimulatory molecule CD4 recognizes MHCII presented peptides resulting in T cell activation (174).

The CD8 T cell subclass, also referred to as CTL (cytotoxic T lymphocytes) undergoes a somewhat similar recognition except they are restricted to peptides presented on MHC class I molecules. MHC I molecules are presented by all cells except red blood cells. MHC I functions to display cellular peptides and this mechanism allows the immune system is to discern self from non-self. If a cell becomes infected, foreign peptide is presented on class I molecules and this targets it for destruction by the CTL. CTL mediated cell killing is similar to NK cells, involving the release of perforin and granzymes to mediate apoptosis.

Investigation into the contribution of T cells in the control of primary *Francisella* infection has been studied. Mice whose CD4 and CD8 T cells are depleted by thymectomy are able to control and partially resolve a primary sublethal intravenous infection with *Ft*. LVS for up to 8 weeks (175). SCID mice (severe combined immunodeficiency, mice lacking T and B lymphocyte development) survive for 20 days, but then die from severe infection by intradermal route. When these mice are grafted with normal T cells, they are able to overcome and clear infection. However, when the graft is from nude mice (athymic mice which lack T cells only), they are unable to do so (170). However, other mechanisms for host resistance are also at play. SCID mice treated with inactivating antibodies against IFN-γ or TNF-α die at 7-8 days post infection. These data indicate that T cell-independent mechanisms are important in early immune responses, while T cell-dependent mechanisms are important later on (170). The specific contributions of different T cell subsets may not be exactly clear. Mice lacking only CD4, CD8 or γδ T cell subsets are able to overcome infection as well as establish immunologic memory as evidenced by resistance to a subsequent *Ft*. LVS challenge (176).

Table 6: CD4 and CD8 T cell subsets

T cell subset	Polarizing Factor	Functions	Secreted cytokines
$CD4^+$			
T_H1 ,	IL-12, IFN-γ	Production of inflammatory cytokines, retards T_H2 development, efficient at intracellular bacterial clearance via macrophage activation	IFN-γ, IL-2, TNF-β
T_H2	IL-4, IL-6	Production of cytokines for adaptive immunity, retards T_H1 development	IL-4, IL-5, IL-10, IL-13.
$CD8^+$			
T_C1	IL-2, IFN-γ and anti-IL-4	Cytotoxicity	IL-2, IFN-γ
T_C2	IL-4	Cytotoxicity	IL-4, IL-5, IL-6, IL-10

CHAPTER 3.6
Myeloid derived suppressor cells

MDSC are a heterogenous group of immature myeloid progenitors cells that originate from the bone marrow. Under normal conditions, immature myeloid progenitors develop into macrophages, dendritic cells or neutrophils. However, in cases of acute or chronic infections, sepsis, trauma or tumor microenvironment their differentiation is inhibited and these cells become suppressive in nature (reviewed in (83,177)). MDSCs were originally identified as an increased cell population in tumor bearing mice and thus, much of the research surrounding MDSC is dedicated to the cancer field. Yet, this cell population is also expanded during acute infections with *Trypanosoma cruzi*, toxoplasmosis, polymicrobial sepsis and *Listeria monocytogenes*, as well as chronic infections with *Leishmania major*, helminths, *Candida albicans* and *Porphyromonas gingivalis* (177). Recently, an expanded population of MDSC was also described in the livers if mice after *Ft*. LVS infection (82).

Activation and expansion of the MDSC cell compartment occurs in response to inflammatory conditions such as those induced by IFN-γ + LPS, IL-1β, IL-6 and Prostaglandin E2 (83). As their name implies, activated MDSCs serve to suppress inflammation by acting on several different cell types. MDSC can inhibit dendritic cell development (178), suppress NK lytic function in patients with hepatocellular carcinoma (179) and suppress T cells by a variety of mechanisms (reviewed in (180)). One such mechanism of T cell suppression is mediated through production of arginase-1 (ARG1), an enzyme that utilizes metabolic L-arginine as its substrate. Depletion of L-arginine

from the local environment subsequently inhibits proliferating T cells and CD3-ζ chain expression, an integral component of the T cell receptor (83).

Cross regulation between many cell types exists during the course of infection. For example, macrophages activated by infection produce cytokines such as IL-12, IL-18 and other cytokines such as IL-15 (interleukin 15) to activate NK cells. Activated NK cells, through IFN-γ production, help to induce a T_H1 balance of $CD4^+$ T cells. In turn, the T_H1 cytokine environment promotes M1 macrophage polarization to amplify the response. Yet, mechanisms must exist to control inflammation and stop the response before detrimental effects of such an inflammatory repertoire affect host tissue. Expansion of suppressive cell types such as MDSC, and the limited life span of neutrophils are two such examples of how inflammation is countered and controlled. Thus, a biological Yin Yang relationship exists within the immune system.

Goals of this Dissertation

A significant body of literature from decades of research has categorized IKKα and IKKβ into specific roles in NF-κB activation. However, it is important to understand how this fits in to an integrated network of responses to get a true idea of the functional meaning. Although IKKα and IKKβ have been described as having different functions in NF-κB signaling, they both, paradoxically, and by separate mechanisms, each have been shown to have anti-inflammatory roles in an *in vivo* bacterial infection model (51,52). The papers describing these roles for IKKα and IKKβ used a model of infection with the extracellular bacteria Group B *Streptococcus*. I questioned whether this would hold true in a different infection model using the intracellular pathogen *Francisella tularensis* LVS.

Francisella spp. target macrophages and neutrophils to down modulate host inflammatory responses to establish a replicative niche. Macrophages and neutrophils have key roles in orchestrating immune responses to pathogens. Apart from their phagocytic abilities, both cell types have a role in controlling bacteria. Macrophages through cytokine production, and neutrophils through production of antimicrobial substances such as ROS and reactive nitrogen help control and modulate immune responses. NF-κB is well known player in host immune response through gene activation. Therefore, does the conditional manipulation of IKKα or IKKβ driven signaling in either of these cell types render the host more or less resistant to the effects of infection?

In this study, I generated a transgenic mouse strain with conditional loss of IKKα or IKKβ in macrophages and neutrophils to study the effects of intradermal bacterial infection with *Francisella tularensis* LVS to address these questions.

Chapter 4
Materials and Methods

Generation of IKK conditional knock out mice:

A targeting vector was designed to introduce loxP sites into *IKK*α or *IKK*β alleles by Lexicon Genetics Inc. (The Woodlands, TX) for our collaborator, Boehringer Ingelheim Pharmaceuticals, Inc. (Danbury, CT), using strategies previously described for p38α floxed mice (181). Briefly, loxP recombination sites were targeted to flank exons 6 and 7 or exons 2 and 3, of the *IKK*α or *IKK*β alleles respectively. Targeting vectors were electroporated into 129/SvEv^{Brd} (Lex-1) ES (embryonic stem) cells for homologous recombination. G418/neomycin resistant ES cell clones were isolated and correctly targeted clones were microinjected into C57BL/6 blastocysts. Chimeric mice were generated by blastocyst injection into pseudopregnant females. The resulting chimeras were mated to C57BL/6 albino females generating heterozygous floxed mice. These mice were then bred to homozygosity to yield the fully floxed strains, $IKK\alpha^{f/f}$ and $IKK\beta^{f/f}$.

Floxed mice were further crossed to the LysM Cre expressing strain (now on deposit with Jackson Laboratories, Bar Harbor, ME, Jax® mouse strain 004781, B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J) to produce $IKK\alpha^{f/f}$*:LysMCre* and $IKK\beta^{f/f}$*:LysMCre* conditional mutants, herein referred to as IKKα or IKKβ cKO (conditional knock outs). The status of each *IKK* allele was confirmed by PCR (polymerase chain reaction).

PCR Genotyping:

Genomic DNA (gDNA) was extracted from mouse tail sections (2-4 mm). Tails were digested in 500 µl of tail digest buffer (0.3% SDS, 50 mM TrisCl pH 7.5, 5 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0, 100 mM NaCl, 5 mM DTT (dithiothreitol) and 5 mM spermidine, all from Sigma-Aldrich) and 100 µg/ml of Proteinase K (Roche Applied Science, Indianapolis, IN) and incubated at 56ºC overnight. DNA was recovered by phenol/chloroform extraction (Roche Applied Science) according to published protocols (182).

PCR samples were set up in a 40 µl reaction containing 1X PCR Buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 1.0 unit of Platinum® Taq DNA polymerase (all from Invitrogen Inc., Carlsbad, CA) and 3 µl of gDNA (~300 ng).

IKKα targeting PCR: 1 cycle at 94ºC for 2 min, then 40 cycles at 94ºC for 30 sec, 60ºC for 30 sec, 72ºC for 45 sec, then 1 cycle at 72ºC for 10 min. Expected sizes: WT 148 bp and IKKα targeted 219 bp. Primers: BI.16.20, BI.16.21.

IKKα Cre-excised PCR: 1 cycle at 94ºC for 2 min, then 40 cycles of 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 45 sec, then 1 cycle at 72ºC for 10 min. Expected sizes: WT 730 bp and IKKα Cre-excised 312 bp. Primers: BI.16-20, BI.16-3.

IKKβ targeting PCR: 1 cycle at 94ºC for 2 min, then 40 cycles of 94ºC for 30 sec, 58ºC for 30 sec, 72ºC for 45 sec, then 1 cycle at 72ºC for 10 min. Expected sizes: WT 285 bp and IKKβ targeted 356 bp. Primers: BI.15-3, BI.15-2.

IKKβ Cre-excised PCR: 1 cycle at 94ºC for 2 min, then 40 cycles of 94ºC for 30 sec, 58ºC for 30 sec, 72ºC for 45 sec, then 1 cycle at 72ºC for 10 min. Expected sizes: WT 259 bp and IKKβ targeted 364 bp. Primers: BI.15-30 (2x), IKKβ-1 (1x) and BI.15.3 (1x).

Targeting and genotyping primer pair sequences are as follows: **BI.16-20**: 5' -ACT CAG GTC ATC AGA CTT GG- 3'; **BI.16-21**: 5' -TAA AGG AAG GTA GGT TGG GG- 3'; **BI.16-3**: 5' -TTC TTC TTA ATC TTC TCA TGC C- 3'; **BI.15-3**: 5' -CAG CGA CAG AGC AAG ATG TG- 3';**BI.15-2**: 5' -TTC TTT GGG CTG AGC TCC TG- 3'; **BI.15-30**: 5' -CAG CCT TTT GAT TTG CAC GC- 3'; **IKKβ-1**: 5' -TGG CAC CCA ATG ATT TGC CAC- 3'.

Animals:

Mice were housed in ventilated cages in a maximum isolation facility equipped with a 12:12 hour light:dark cycle and fed a standard chow diet and sterile water ad libitum. All procedures were performed in accordance with State University of New York at Stony Brook IACUC protocols.

All mice referred to as "WT" (wild-type) in these studies are of the respective parental IKK$^{flox/flox}$ strain.

Derivation of bone marrow macrophages (BMDM):

Bone marrow derived macrophages (BMDM) were generated according to published protocols (183). Briefly, mice were euthanized by CO_2 asphyxiation and both femurs were aseptically removed. The epiphyses were cut from the ends of each femur and the bone was flushed using a syringe fitted with a 27-gauge needle filled with 5 ml of base media per femur (RPMI 1640, 10% FBS (fetal bovine serum), 1% penicillin/streptomycin (all purchased from Gibco/Invitrogen, Carlsbad, CA). The cells from 2 femurs were pooled and placed in a 15 ml collection tube (Fisher Scientific,

Pittsburgh, PA). To remove large debris, the tube was inverted several times to suspend the cells and then allowed to sit for precisely 60 seconds. The cell-containing media was recovered and placed in a fresh tube, avoiding collection of the settled debris. Cells were pelleted by centrifugation at 300 x *g* for 5 minutes at 4ºC. To lyse red blood cells, the pellet was resuspended in 10 ml of ACK hypotonic buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, all purchased from Sigma-Aldrich, St. Louis, MO), mixed well and allow to stand at room temperature for 5 minutes. Cells were pelleted as above, washed once in PBS and resuspended in 10 ml of macrophage growth medium (base media supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine from Gibco/Invitrogen, Carlsbad, CA and 30% L-cell conditioned media as a source of M-CSF, macrophage colony stimulating factor (184,185) and plated in a 10 cm non-tissue culture treated dish (Corning Inc., Corning, NY). The plate was incubated at 37ºC with 5% CO_2 overnight. The following day, cells were triturated and divided into two plates at a final volume of 10 ml. The media was replaced on day 3. Adherent macrophages were collected on day 5 by removing the growth media and adding 5ml of ice cold PBS and incubating at 4ºC for 10 minutes. The cells were collected and the plates were rinsed with an additional 5 ml of ice cold PBS. The cells were centrifuged as above and the cell pellet was snap-frozen in LN_2 (liquid nitrogen) and stored at -80ºC until use for immunoblot assays.

<u>*Neutrophil isolation:*</u>

Bone marrow cells were isolated from mouse femurs and tibias in a similar fashion as described above for macrophages and then neutrophils were separated out according to

published protocols (186). Briefly, cells were spun down at 400 x *g* and resuspended in 1 ml of HBSS-EDTA (1x Hanks Balanced Salt Solution, Gibco/Invitrogen, Carlsbad, CA, 15 mM EDTA (Sigma-Aldrich, St. Louis, MO). A three-layer density gradient was set up as follows, from the bottom to top: 78%, 69% and 52% Percoll™ (GE Healthcare, Piscataway, NJ, with 100% Percoll™ being equal to 9 parts Percoll™ and 1 part 10x HBSS). Cells were layered on top of the gradient and then centrifuged at 1500 x *g* for 30 minutes at room temperature without braking. Neutrophils were collected from the 69%/78% interface. Purified neutrophils were washed once in 1x HBSS-EDTA, a pellet was snap frozen in LN_2 and stored at -80ºC until use for immunoblot assay. A portion of cells were set aside and stained with Gr-1 antibody and subjected to flow cytometry. It determined by high Gr-1 staining the yield was 70-80% purity.

Immunoblotting:

Frozen cell pellets were briefly thawed on ice, and then resuspended in 200-500 µl of Triton-X lysis buffer (1% Triton-X100, 100 mM Tris-Cl pH7.6, 150 mM NaCl supplemented with 1 µg/ml Leupeptin, 1µg/ml Pepstatin and 100 µg/ml PMSF, all from Sigma-Aldrich, St. Louis, MO). The lysates were vortexed briefly and DNA was sheared by passing the samples through a 1 ml tuberculin-syringe, fitted first with a 20-gauge needle and then through a 27-gauge needle, 5-7 times each. The lysates were then centrifuged at 14,000 x *g* for 2 minutes and supernatants were placed in a fresh tube. Protein quantification of the solution was determined using the BioRad Protein Assay Kit (BioRad, Hercules, CA) as per manufacturer's instructions and the absorbance at 595 nm was taken on a spectrophotometer. The final concentration was derived from comparison

with known concentrations of BSA (bovine serum albumin, Sigma-Aldrich, St. Louis, MO) assayed in the same way and fitted to a standard curve. To prepare samples for immunoblotting, 30 µg of lysate were mixed with 1X Laemmli Buffer (BioRad), 0.3% β-mercaptoethanol and H_2O to a final volume of 30 µl. The samples were boiled at 100ºC for 5 minutes, briefly spun and then resolved by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) on a 10% gel and transferred to PVDF membranes (Polyvinylidene fluoride, Millipore, Inc. Billerica, MA) following published protocols (182). Each membrane was blocked in 1x blocking buffer (5% non-fat dried milk, 25 mM Tris, pH 7.5, 150 mM NaCl) for one hour. Membranes were then incubated in 10 ml of rabbit poly-clonal primary antibodies against mouse IKKα, IKKβ (both from Cell Signaling Technology, Inc., Boston, MA) or β-actin (Sigma-Aldrich), diluted 1:1000 in blocking buffer plus 0.05% Tween-20 (Sigma-Aldrich) and incubated overnight at 4ºC with gentle rotation. Membranes were washed 2x for 15 minutes each in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20), 1X in TBS (25 mM Tris, pH 7.5, 150 mM NaCl), blocked for 15 minutes in blocking buffer and then placed in 10 ml of donkey anti-rabbit-HRP (horseradish peroxidase) secondary antibody (Amersham/GE Healthcare, Piscataway, NJ) diluted 1:2000 in blocking buffer plus 0.05% Tween-20, for 1 hour. The membranes were washed as above and developed using the Lumi-Light Plus Western detection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

Infection Model:

Eight- to twenty-week old female mice were injected i.d. (intradermal) at the base of the tail in a total volume of 100 µl containing 10^8 CFU of *Ft.* LVS for survival studies, or 10^6 CFU for all other assays unless otherwise noted.

Bacteria:

Ft. LVS (ATCC 29684, American Type Culture Collection, Manassas, VA) were grown in the laboratory of Dr. Martha Furie, according to published protocols (187). Briefly, frozen stocks were streaked on CHA II plates and grown at 37ºC. On day 3, single colonies were inoculated into prewarmed Mueller Hinton II broth (BD Worldwide, Franklin Lakes, NJ) at 37ºC, with shaking for 16-18 hours. Cultures were grown to an optical density at 600 nm (OD_{600}) between 0.2-0.4 and diluted in sterile PBS to obtain the specified inoculation dose.

Retroplate assay:

Inoculation doses were verified by colony plate counts. Briefly, the bacterial inoculum was serially diluted three times in sterile PBS (Phosphate buffered saline, Gibco/Invitrogen, Carlsbad, CA), neat homogenates and serial dilutions were plated on Chocolate II agar with Hemoglobin and IsoVitaleX (CHA II, BD Worldwide, Franklin Lakes, NJ). Plates were incubated at 37ºC for three days and the resulting colonies were counted. Calculations to derive colony counts per ml were made as follows:

Equation 1:

# of counted colonies X dilution factor X volume factor = colony count

where the dilution factor is the fold-dilution from serially diluted homogenates and the volume factor is the volume of homogenate plated (10 or 100 µl) divided by 1 ml. For organ burden assays, this number was further divided by the liver factor, which is the weight in grams of the liver section used in the assay (generally ~ 0.1-0.2g) divided by the total liver weight.

Equation 2:

$$\frac{\text{colony count}}{\text{liver factor}} = \text{Liver colony count}$$

Analysis of the median lethal dose, LD_{50}:

The median lethal dose was estimated in WT mice after i.d. injection of 10^7 or 10^8 CFU (colony forming units) of *Ft*. LVS and monitoring survival for 15 days. Actual bacterial counts injected were determined by plate counting as described above.

Survival Analysis:

Mice (10-15 per group) were injected with 10^8 CFU of *Ft.* LVS i.d. and monitored twice daily for survival over a period of 14 days. Kaplan-Meier survival curves were generated and their relative mortalities in response to lethal infection were compared by a log-rank test. Additionally, long-term survival at sublethal doses was tested. Mice (n = 6 per group) were injected with 10^7 or 10^6 CFU of *Ft.* LVS i.d. and monitored for survival over a period of 90 days.

Organ Burden determination:

Four to five mice per group were injected with 10^6 CFU of *Ft*. LVS i.d. At the indicated time points, mice were euthanized and their livers were aseptically removed. A section of the median lobe (~ 0.1-0.2 g) was placed in a pre-weighed tube containing 1 ml of sterile PBS. The tube containing the liver section and the remainder of the liver were weighed and recorded. The liver section was homogenized in 1 ml of sterile PBS in a stomacher bag (Fisher Scientific, Pittsburgh, PA). Homogenates were serially diluted and both neat homogenates and serial dilutions were plated and incubated for 3 days at 37ºC. Colonies were counted as described in the retroplate assay in order to determine the actual number of CFU per liver.

Histology:

Mice were injected i.d. with a sublethal dose of 10^6 *Ft*. LVS. At the indicated time points, organs were removed and fixed in 10% neutral buffered formalin (Sigma Diagnotics, St. Louis, MO) and embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin, dehydrated in graded alcohols, cleared with xylene (Fisher Scientific, Pittsburgh, PA) and mounted with Acrymount (EMS, Hatfield, PA). Hepatic granulomas from 4 mice/group were quantified by examination of 10 fields/liver under 200x magnification and photographed on an Olympus BX41 light microscope (Olympus, Tokyo, Japan).

Non-parenchymal cell (NPC) isolation:

Mice were injected with 10^6 CFU i.d. of *Ft.* LVS. On the day of harvest, livers were perfused with 1 mM citrate (Sigma, St. Louis, MO) dissolved in Hanks Buffered Saline Solution (HBSS, Gibco/Invitrogen, Carlsbad, CA). Livers were placed in digest buffer containing 0.05% Collagenase II, 0.002% DNase 1 and 1% BSA (bovine serum albumin, all from Sigma-Aldrich, St. Louis, MO) in HBSS, at 37ºC for 30-45 minutes. Digests were filtered through a 70 µm cell strainer (BD Falcon, San Jose, CA). Red blood cells were lysed in ACK hypotonic buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). NPCs were then isolated by a flotation method using Optiprep density gradient medium (AxisShield, Oslo, Norway) according to the manufacturer's protocol C-24. Upper and lower isolates were collected and the NPC fraction was confirmed by flow cytometry. The upper isolate was >97% $CD45^+$ (eBiosciences, San Diego, CA).

Flow Cytometry:

Fcγ receptors (fragment, crystallizable, gamma receptor) were blocked with CD16/32 Antibody (eBiosciences, San Diego, CA). Surface epitopes were stained with antibody for 30 min. (Table 7), washed 2 times with FACS Stain Buffer (FSB, 1% heat inactivated FBS (Fetal bovine serum, Gibco/Invitrogen, Carlsbad, CA), 0.09% w/v NaN_3 in PBS). For experiments with intracellular antigens, cells were incubated with GolgiPlug (BD Biosciences, San Jose, CA) for 6 hours according to manufacturer's protocol prior to fixation/permeabilization to prevent cytokine release. Cells were fixed in Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA) for 20 min. then washed 2

times in Perm/Wash (as supplied in the kit). Antibodies to intracellular antigens were added to cells and incubated for 30 min. Next, cells were washed twice in Perm/Wash and resuspended in FSB. Unlabeled antibodies required labeling with an additional secondary antibody containing a fluorophore. After intracellular labeling, secondary antibodies were added, incubated for an additional 30 min., washed 2 times in Perm/Wash and fixed in a 1% PFA /PBS solution (paraformaldehyde, Sigma Diagnotics, St. Louis, MO). All labeling and permeabilization steps took place in the dark with rotation. Acquisition was performed on a FacsCalibur instrument (BD Biosciences, San Jose, CA), and data was analyzed using FlowJo V9.0.1 (TreeStar, Ashland, OR).

Statistical analysis:

Log rank and one-way ANOVA (analysis of variance) and Tukey's post-test were performed using Graphpad Prism5 (GraphPad Software, Inc., La Jolla, CA).

Table 7: Antibodies used in flow cytometry

Antibody	Clone	Fluorophore	Supplier
Arg-1	Goat polyclonal	Unconjugated	Everest Biotech
CD3α	B111922	PE	BioLegend
CD3ε	145-2C11	FITC	eBbiosciences
CD4	RM4-5	FITC	BioLegend
CD8α	53-6.7	FITC	BioLegend
CD11b	M1/70	PerCP Cy5.5	BD
CD11b	M1/70	PE	BioLegend
CD45	30.F11	FITC	BioLegend
F4/80	BM8	FITC	eBbiosciences
Ft. LPS	Rabbit Polyclonal	Unconjugated	Generous gift of Dr. Jorge Benach
IFN-γ	XMG1.2	APC	BD
IL10	JES5-16E3	PE	BD
IL12 p40/70	C15.6	APC	BD
pan-Neutrophils/Ly-6B.2	7/4	FITC	Serotec
NK1.1	PK136	PerCP Cy5.5	BD
RELMα/FIZZ1	Rabbit polyclonal	Unconjugated	Abcam
Donkey Anti-rabbit	-	PE	Abcam
Donkey Anti-goat	-	PE	Abcam

Chapter 5
Results

Generation of IKKα and IKKβ cKO mice:

Gene targeting in mice was done using the Cre-loxP recombination method (188). This method involves the insertion of two 34 bp (base pair) DNA sequences, called loxP sites, flanking the portion of the gene to be deleted. Genes flanked in this way are referred to as "floxed" genes. These sites are recognized by P1 bacteriophage Cre recombinase, a Type I topoisomerase which, upon its expression, mediates the excision of the intervening DNA. Targeting vectors were designed to position loxP sites in the gene region containing exons 6 and 7 for *IKKα* and exons 2 and 3 for *IKKβ* (Figure 4A & E). LoxP containing vectors were electroporated into ES cells and selected after homologous recombination (Figure 4B & F). Positive blastocysts were injected into pseudopregnant mice and backcrossed to generate $IKK\alpha^{f/f}$ or $IKK\beta^{f/f}$ floxed mice (Figure 4C & G).

LysM Cre mice express P1 cre recombinase under the control of the M-lysozyme promoter in mature macrophages and neutrophils mediating conditional deletion of floxed genes specifically in these cell types (189). The resulting cre-mediated deletions within each locus are shown in Figure 4D and H. Resulting mice were viable, fertile and did not exhibit any morphological abnormalities or behaviors.

Figure 4

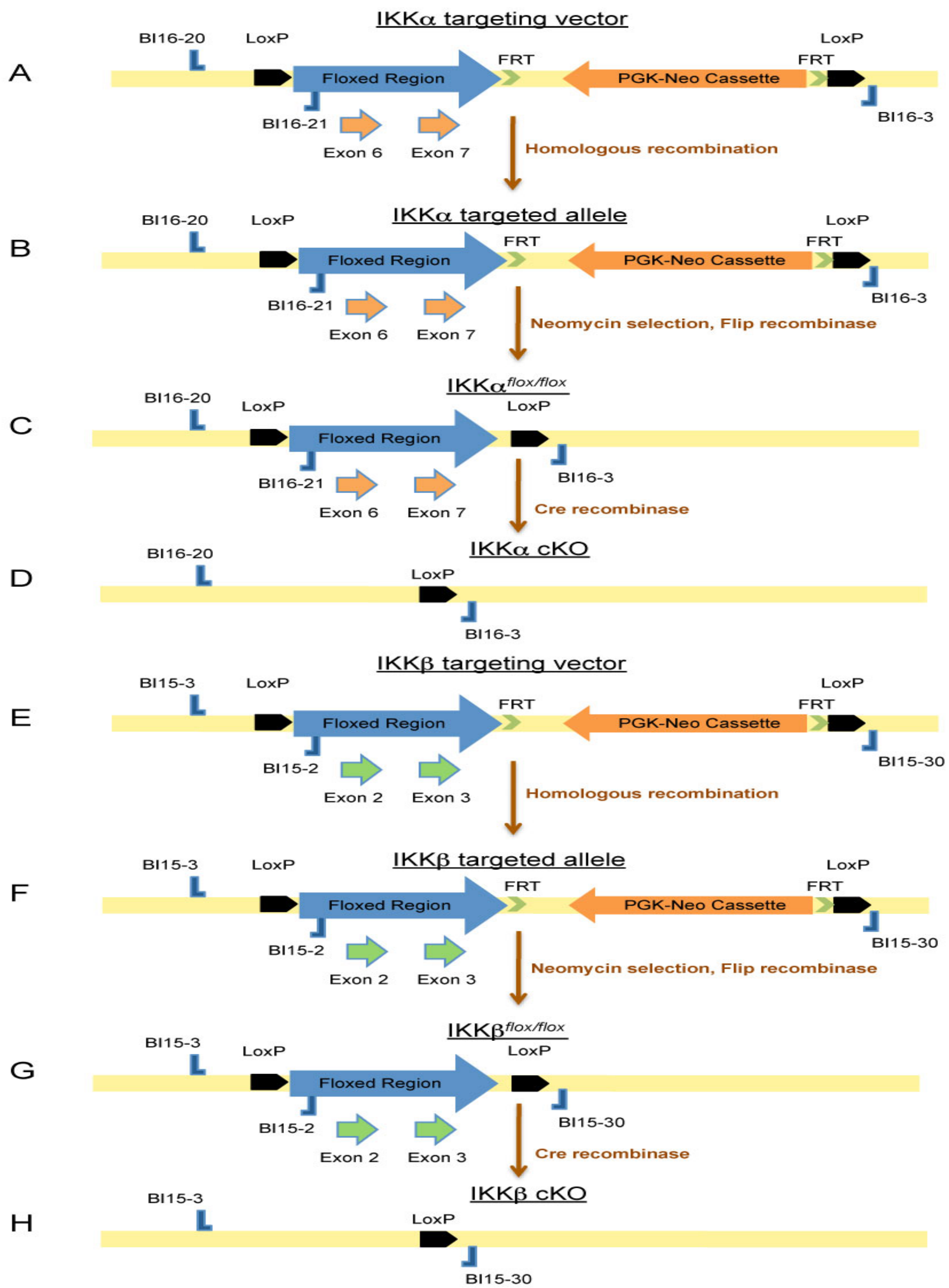


Figure 4: Generation of *IKK*α and *IKK*β conditional knock out mice: A schematic showing the generation of floxed *IKK*α (A-D) and *IKK*β (E-H) alleles. **A. & E.** Targeting vectors were generated against *IKK*α and *IKK*β for homologous recombination in ES cells, and **B. & F.** resulting alleles are shown, respectively. **C.** *IKK*α floxed alleles encompass exons 6 and 7 and **G.** *IKK*β floxed alleles encompass exons 2 and 3. **D. & H.** Show the genomic contig after Cre-recombinase expression in *IKK*$\alpha^{flox/flox}$ and *IKK*$\beta^{flox/flox}$ mice, respectively.

Validation of conditional IKK knock outs:

In order to verify that each of the IKKs was appropriately deleted in the specified cell types, immunoblotting was performed on whole cell extracts from bone marrow derived macrophages and neutrophils (Figure 5A-B). As expected, IKKα and IKKβ cKO mice exhibited virtually no expression of either kinase in the appropriate strains. Some residual IKKβ was detected in isolated neutrophils and this is likely due to the 70-80% purity of the recovered mature neutrophils.

Figure 5

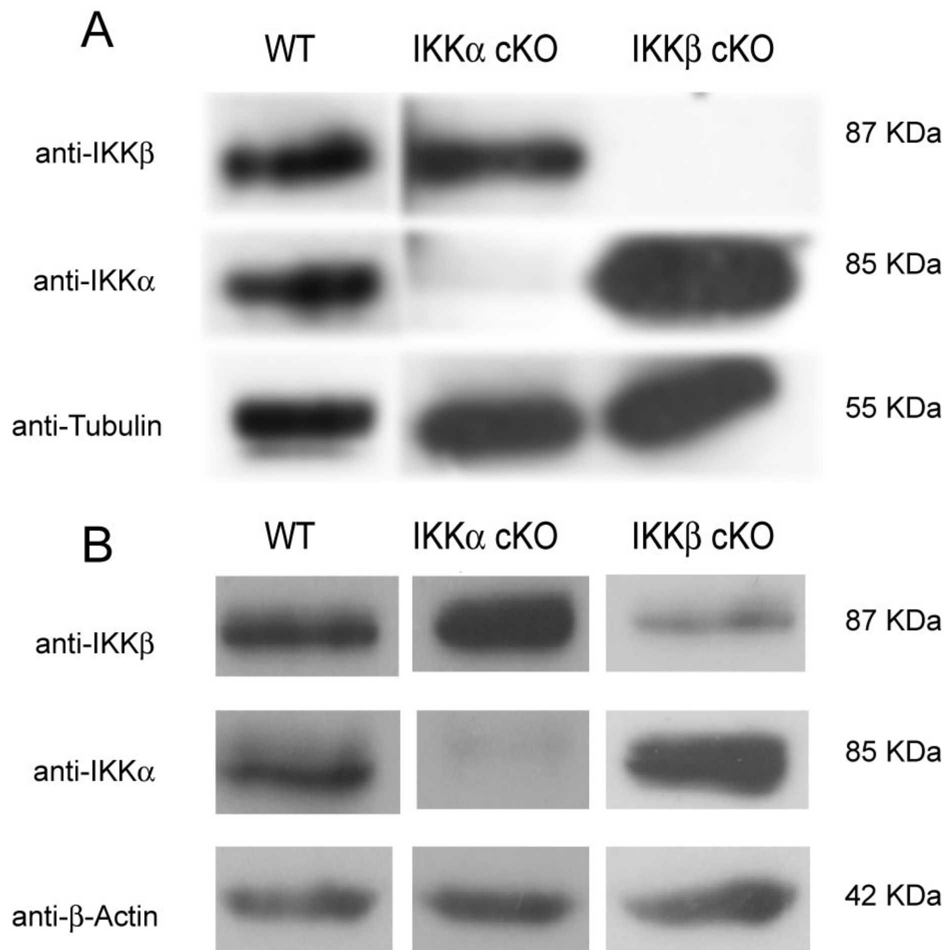


Figure 5: Conditional IKK expression in macrophages and neutrophils: Immunoblot analysis of IKK expression in whole cell extracts of **A**. five day *in vitro* derived bone marrow macrophages (190) or **B.** bone marrow purified neutrophils from WT, IKKα cKO and IKKβ cKO mice. (Neutrophil immunoblot is courtesy of David Habiel)

Determination of the LD_{50}:

In order to test the roles of IKKα or IKKβ in a *Ft*. LVS infection, I first had to establish the lethal and non-lethal doses. Classically, lethality/toxicity testing involves the determination of the LD_{50} (median lethal dose). Precise measurements of the LD_{50} generally require ~100 animals, and testing of multiple (5 or more) dosages. Alternative methods have been described to obtain the LD_{50} using fewer animals (191). The C57BL/6 mouse strain tends to be more resistant to *Ft*. infection according to published data (63,192,193). For example, an i.p. (intraperitoneal) injection, an artificial but sensitive route of delivery, resistance to infection in different strains of mice is as follows: C57BL/6 (2.7) > C3H/HeJ (1.5) >BALB/c (0), where the number in parentheses represents the Log_{10} of the LD_{50} (63,192,193). In an intradermal route of infection, the LD_{50} of C57BL/6 mice is reported to be greater than 10^6 CFU of bacteria (192,193). Taking these reports into consideration, a small sampling population of WT (IKK floxed) mice was used to confirm the LD_{50} by intradermal injection. WT mice were tested at two doses of *Ft*. LVS, 10^7 and 10^8, for 15 days. Figure 6 shows that nearly 80% of mice survive an i.d. infection with 10^7 bacteria while only ~20% survive at a dose of 10^8. Actual bacterial doses were quantified by a retroplate assay and the estimated LD_{50} was determined to be 10^8 CFU of *Ft*. LVS per animal.

Figure 6

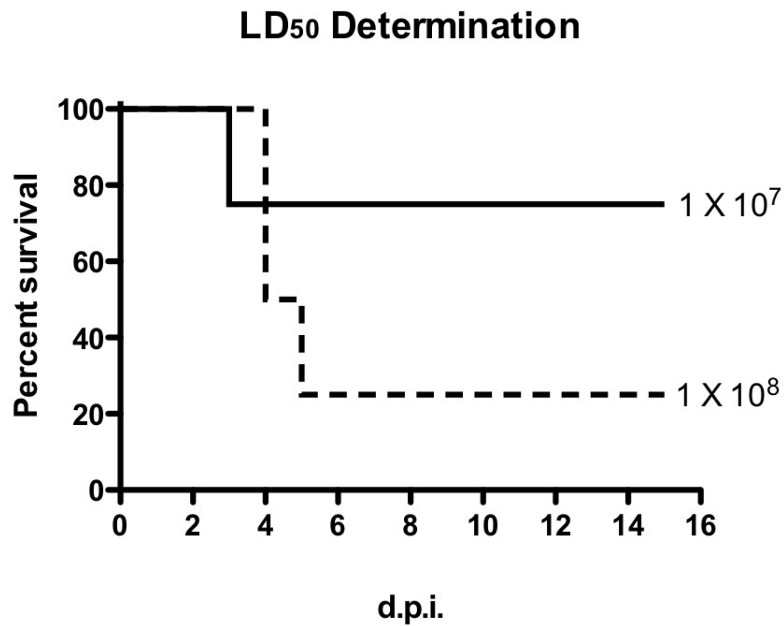


Figure 6: Dosage effects on survival after *Ft.* LVS infection in wild type mice: An assay to determine the median survival dose was performed on small groups (n=4) of WT (IKK$^{flox/flox}$) mice. Two doses of bacteria, 10^7 and 10^8 were tested by intradermal infection. Actual bacterial counts were determined by retroplate assay and LD_{50} was estimated to be 10^8 CFU of *Ft.* LVS per animal.

Susceptibility of IKKβ cKO mice to intradermal *Ft.* LVS infection:

To investigate the roles of the IKK kinases in host response to *Ft.* LVS infection, IKKα cKO and IKKβ cKO mice were injected with the LD_{50} dose of 10^8 CFU i.d. and scored for their relative survival rates. All IKKβ cKO, but not WT or IKKα cKO, mice, succumbed to infection by day 10 (Figure 7A). A comparative Log-rank test P value of 0.0014 shows this difference was significant, yet no distinct differences in the survivals of WT and IKKα cKO mice were seen. Additionally, mice were tested at sublethal doses of 10^7 (Figure 7B) and 10^6 (Figure 7C) for their long-term survival over a course of 90 days and mice from all three strains had similar survivals at these doses.

These results indicate that IKKβ function in macrophages and neutrophils is necessary for host survival during the initial phases of infection (10 days, Figure 7A) at a lethal dose. The LD_{50} for IKKβ falls in a narrow range of inoculation doses, between 10^7 (~ 97% survival, Figure 7B) and 10^8 (100% mortality, Figure 7A). Due to the similarities of IKKβ cKO and WT mice in survival at sublethal doses, (Figures 7B-C), it can be concluded that IKKβ function is required in macrophages and neutrophils only when infection reaches a high, overwhelming dose. Additionally, these data confirm the estimation of the WT LD_{50} value of 10^8 and show that the LD_{50} for WT and IKKα are comparable. Therefore, IKKα in macrophages and neutrophils is dispensable for host survival in this infection model.

Figure 7

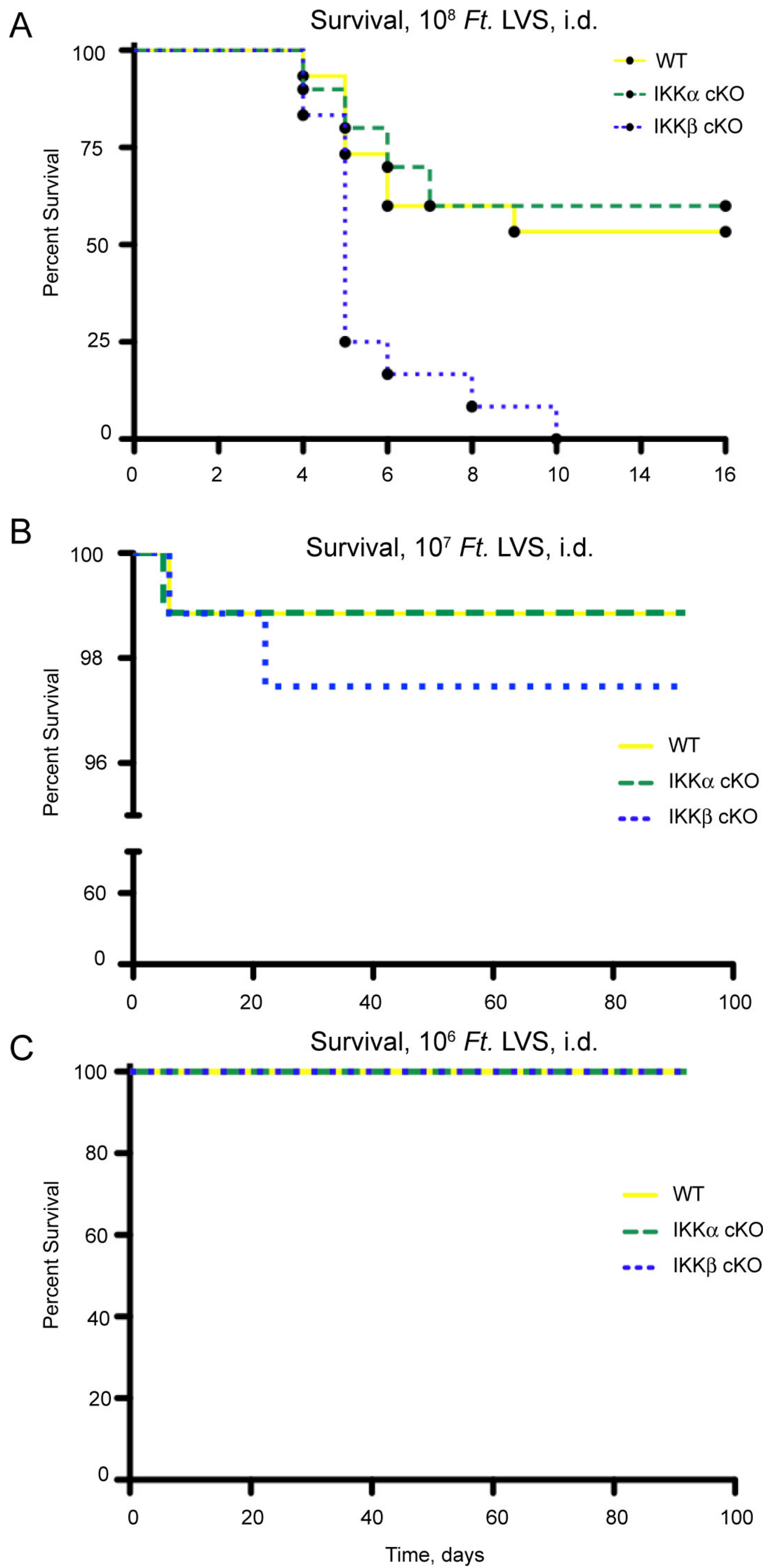
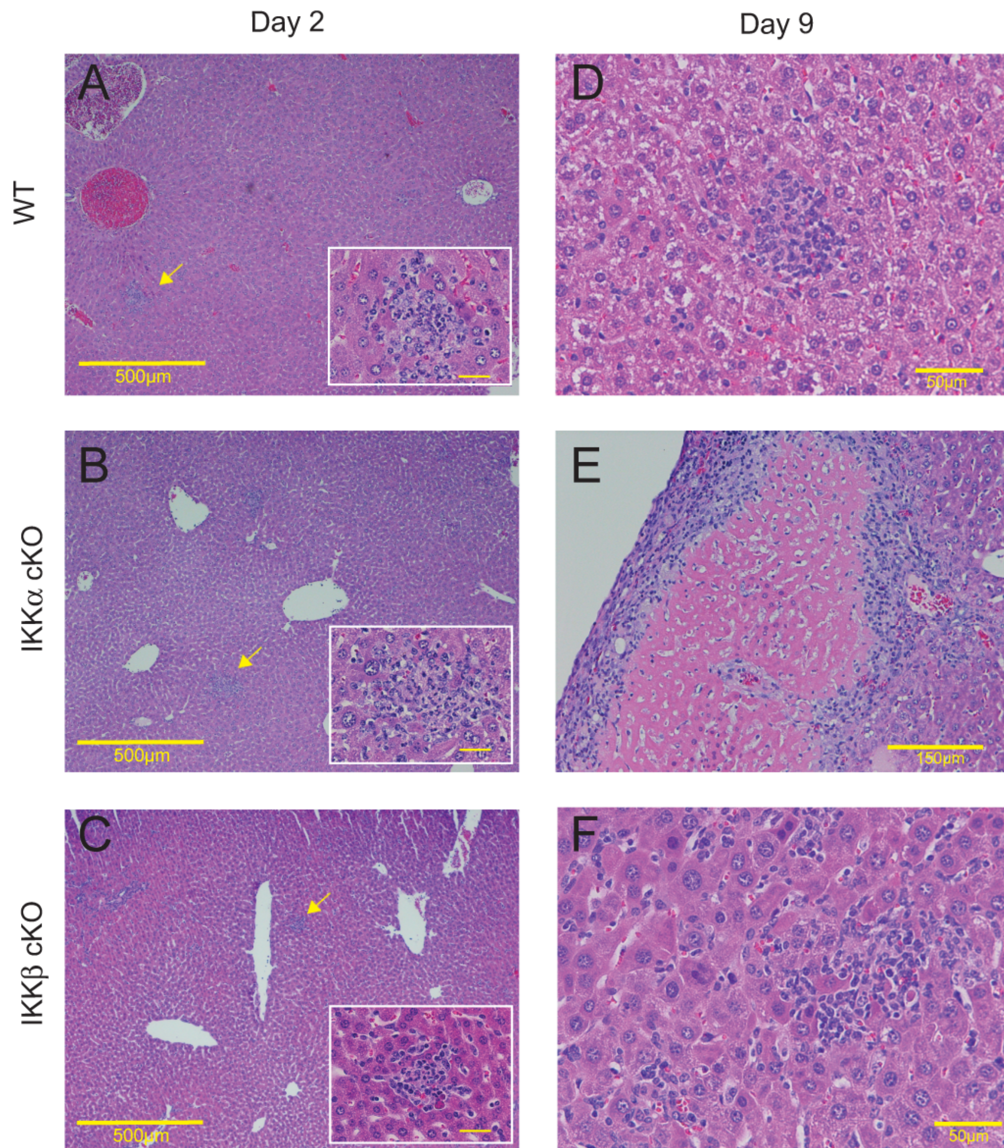


Figure 7: IKKβ, but not IKKα, function in macrophages and neutrophils is essential for host survival to a lethal, i.d. *Ft.* LVS infection. Kaplan-Meier survival plots for WT, IKKα cKO and IKKβ cKO mice after i.d. injection with **A**. 10^8 (n=10-15 mice per group, Log-rank P value = 0.0014). Long term survival at sublethal doses of **B.** 10^7 (n=6 mice per group) or **C.** 10^6 CFU *Ft*. LVS (n=6 mice per group) were also tested showing that differences in IKKβ lethality occurs only at overwhelming doses of bacteria.

Loss of either IKKα or IKKβ signaling in macrophages causes a defective granulomatous response:

Next, I asked if conditional loss of either IKK affected disease progression in a sub-lethal model. The liver undergoes a pronounced inflammatory reaction upon *Ft.* LVS infection, which is characterized by the development of granuloma-like structures (68,81,82). Histological examinations of livers from mice infected i.d. with 10^6 CFU at early and late time points were evaluated for granuloma formation and disease progression. Granuloma formation was evident at two days post-infection in all three strains (Figures 8 A-C). While minor morphological differences in granulomas were noted at day 2, remarkable differences in granuloma progression were observed by day 9. Compared to WT animals, livers of IKKα cKO mice developed fewer granulomas (Figure 8G). However, several of these granulomas became abnormally large and macroscopically discernible (Figure 8D). These large granulomas contained a central necrotic core (note that panel E magnification is 100x while D and F is 200x). In contrast, hepatic granuloma formation was defective in *Ft.* LVS infected IKKβ cKO mice, as evidenced by their improperly nucleated structures by day 9. These granulomas were less compact than WT granulomas (compare figures 8 D and 8 F) and showed a wide disbursement of inflammatory cells (Figure 8F). Furthermore, IKKβ cKO granulomas were more numerous at both time points tested (Figure 8G).

Figure 8



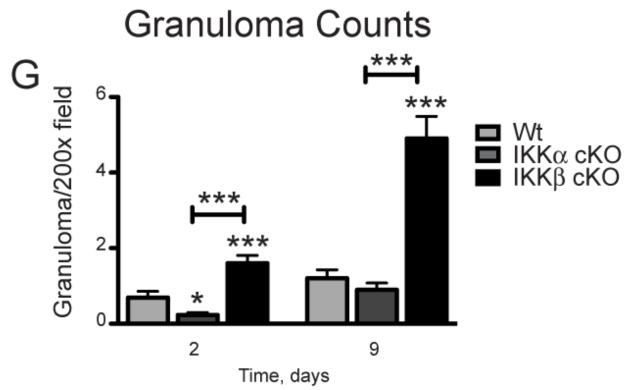


Figure 8: Loss of either IKK kinase causes defects in granuloma development. Early (day 2) and late (day 9) granuloma development in **A & D,** Wild Type (WT), **B & E**, IKKα cKO and **C & F**, IKKβ cKO mice infected i.d. with 10^6 *Ft*. LVS. Panels A, C and E are shown at 40x magnification and scale bars represent 500 µm. Representative granulomas, identified by yellow arrows, are shown as insets at 400x magnification, where scale bars represent 30 µm. Panels D and F are 200x magnification with a scale bar representing 50 µm. Panel E is a lower magnification (100X) with a scale bar representing 150 µm to accommodate the large granulomas found in IKKα cKO livers. **G.** Granulomas were quantified as counts per 200x field and the data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistics are represented as * = $P < 0.05$ and *** = $P < 0.0001$. Data was averaged from 10 fields per section from 4 mice per group.

IKKβ cKO mice have increased bacterial colonization of the liver.

In order to determine if the observed granuloma defects were due to increased bacterial colonization, a time course assay to determine the liver burden was performed on liver homogenates from mice infected with a sublethal dose of 10^6 *Ft*. LVS (Figure 9A). IKKβ cKO mice uniquely presented a higher degree of liver colonization, while IKKα cKO infected mice were similar to WT. Since macrophages are considered a primary cellular target of *Francisella* infection (59,82,193) and because the conditional knock outs occur in macrophages, I questioned whether the macrophages in these strains were comparably infected. To address this question, non-parenchymal cells were isolated from the livers of infected mice and analyzed by flow cytometry. Cells were first stained with F4/80 and CD11b antibodies to define the macrophage population and then cells were intracellularly stained with an antibody directed against *Ft*. LPS to determine the number of *Ft.* containing cells. IKKβ cKO infected mice showed modest increases in the number of *Ft*. positive macrophages on days 2 and 6, and this only reached statistical significance on day 6. In spite of these small increases, the number of *Ft*. positive macrophages throughout the 8-day time course was overall similar to those seen in WT and IKKα cKO mice (Figure 9B).

Bacteria are first detectable in the livers at days 2-3 post infection and this coincides with the appearance of granulomas (68,81,82), indicating this is when colonization is established. In later time points during the course of infection, increases in bacterial counts are more representative of bacterial growth and reinfection of other resident cells. Notably, all strains of mice showed similar numbers of *Ft*. positive

macrophages at day 2 (Figure 9B). This implies that an increased infection of macrophages is not the cause of increased bacterial colonization of the livers in IKKβ cKO mice, and thus, the increased burden represents infection of other cell types in the liver. The liver burden in IKKβ cKO mice was also significantly elevated through day 6, and still modestly elevated at day 14. Together, these data indicate that macrophage and neutrophil IKKβ functions to suppress bacterial colonization and control growth in the liver.

Figure 9

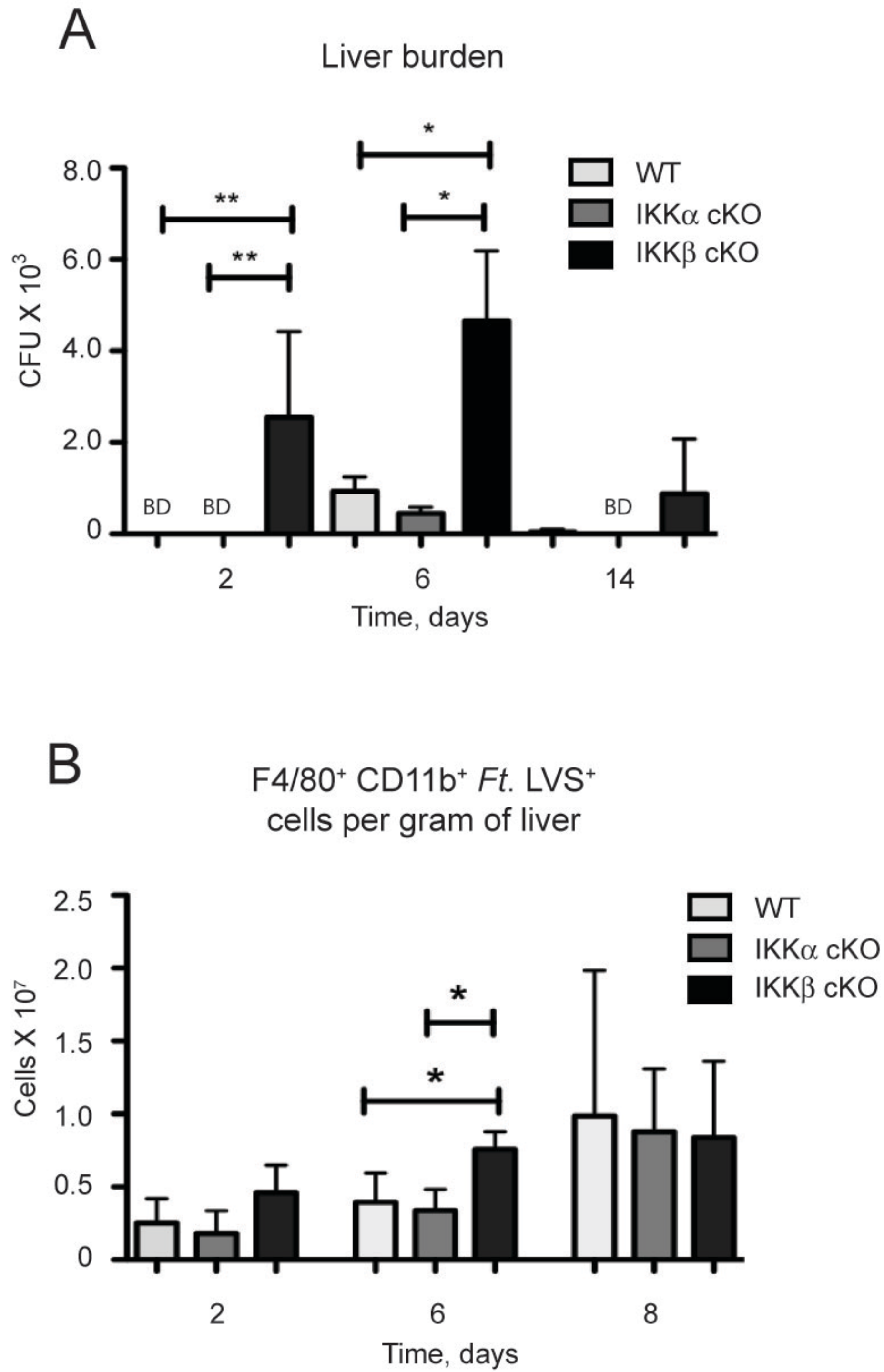


Figure 9: Increased *Ft.* LVS colonization in IKKβ cKO livers.
A. Bacteria were titred from liver homogenates the indicated time points after injection with 10^6 CFU *Ft.* LVS. **B.** Non-parenchymal cells were isolated from infected livers and flow cytometry was used to assay for F4/80/CD11b/*Ft*. LPS^+ cells to determine the extent of macrophage infection. P values were determined by one-way ANOVA with Tukey's ad hoc post test * = $P < 0.05$. N= 3-6 mice per group. BD, below detectable limits.

***Ft.* LVS infected livers of IKKβ cKO, but not IKKα cKO mice, show evidence of M1 and M2 macrophage activation**

Macrophages are a primary target of *Ft*. infection and a major component of *Ft*.-induced granulomas (59,82,193). The differential effects of IKK signaling in macrophages on granuloma formation could be due to intrinsic macrophage functions or extrinsic effects that macrophages and/or neutrophils may have on other immune effector cells. To look into these possibilities, I questioned whether the defects seen in granulomas from conditional knock out mice were the result of differential M1/M2 activation programs or a disordered accumulation of other specific cell types in the infected livers. Since altered granuloma formation became most evident by day 9, I used flow cytometry to quantitatively evaluate the cellular constituents of hepatic non-parenchymal cells (NPCs) leading up to that point. Macrophages were identified by their F4/80 expression in conjunction with macrosialin (murine CD68) or the mannose receptor (CD206), respectively to define M1 (Figure 10A-B) and M2a (Figure 11A-D) macrophage subtypes.

M1 and M2a macrophages are defined by differential expression patterns that are considered either proinflammatory or anti-inflammatory/wound-healing respectively. Both M1 and M2a macrophage subtypes were present in all livers. This observation is not unexpected, because in an *in vivo* environment, cells are not all infected at the same rate, making macrophage polarization an ongoing process. Proinflammatory M1 macrophages were increased in IKKβ cKO livers at day 8 post-infection relative to WT mice (Figure 10A, fold change = 3.67). However, this increase did not correspond to an increase in IL-12p70 activation at the same time point (Figure 10B). IKKβ cKO did show increased

cellular activation of IL-12p70 on day 2 with respect to WT or IKKα cKO mice (Figure 10B, IKKβ cKO vs. WT fold change = 5.04, IKKβ cKO vs IKKα cKO fold change = 2.27), indicating an early, heightened proinflammatory state. However, this effect dissipated throughout the remainder of the time course.

Modest increases in the number of $CD206^+$ macrophages were seen in both IKKα and IKKβ cKO mice at all time points tested (Figure 11A). However, on day 6, only IKKβ cKO mice showed a significant shift toward M2a macrophage polarization as evidenced by the expression of several M2 markers: IL-10 (Interleukin 10, Figure 11B. Day 6 IKKβ cKO vs. WT fold change = 2.00; Day 6 IKKβ cKO vs. IKKα cKO fold change = 2.00), Fizz-1 (Found in inflammatory zone-1, Figure 11C. Day 6 IKKβ cKO vs. WT fold change = 2.70; Day 6 IKKβ cKO vs. IKKα cKO fold change = 2.40), and Arg-1 (Arginase 1, Figure 11D. Day 6 IKKβ cKO vs. WT fold change = 3.84; Day 6 IKKβ cKO vs. IKKα cKO fold change = 3.24). Taken together, these data indicate that IKKβ cKO mice develop an early proinflammatory state in M1 macrophages on day 2 (Figures 10A & B) which is subsequently compensated by a shift towards M2a macrophages on day 6. It also follows that IKKβ acts to suppress polarization of these subtypes in *Ft.* LVS infected mice. Conversely, IKKα cKO mice did not show any shifts in macrophage polarization.

Figure 10

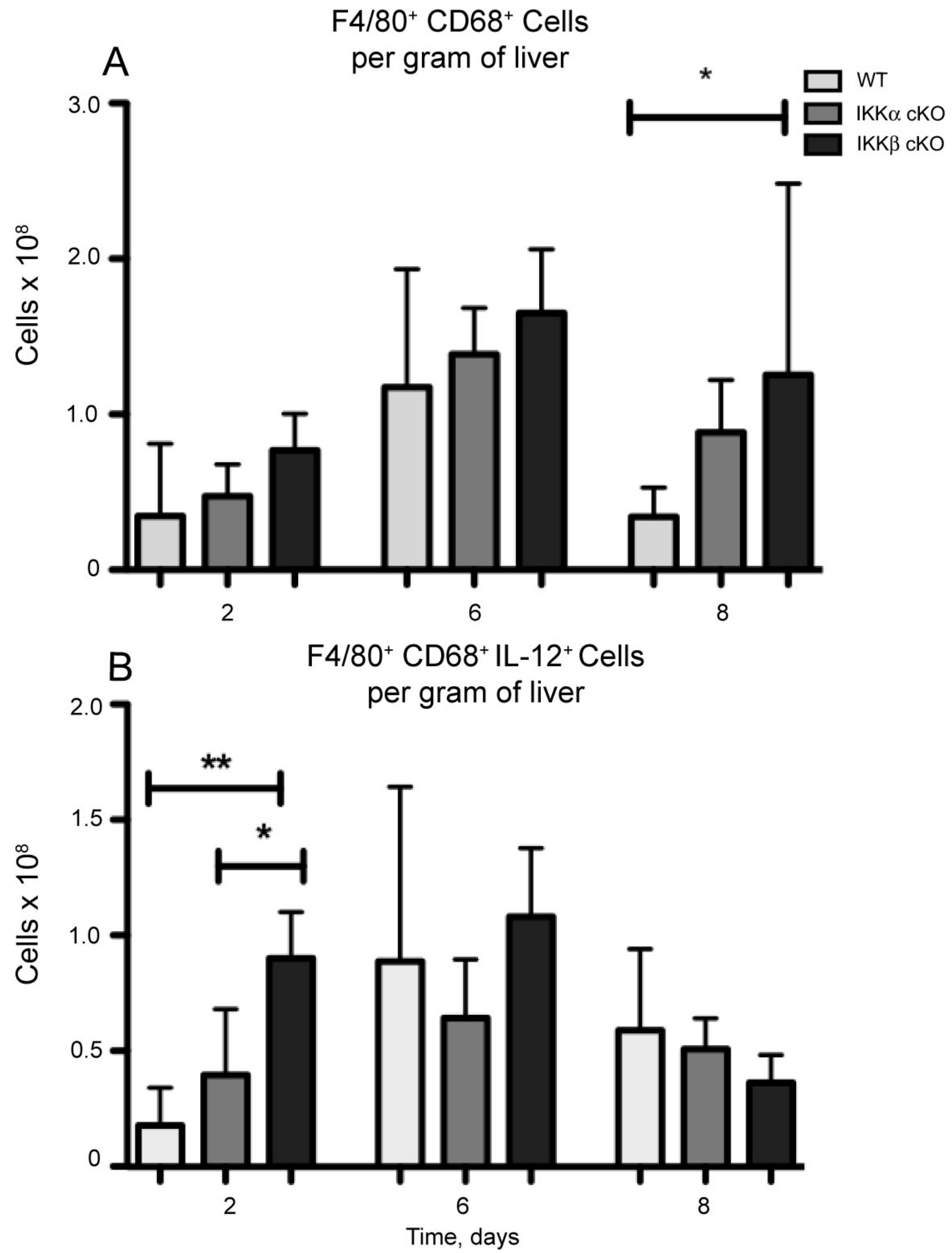


Figure 10: Proinflammatory M1 macrophage activation in IKKβ cKO mice by *Ft.* LVS intradermal infection: Hepatic NPCs were isolated from WT, IKKα and IKKβ cKO mice infected i.d. with 1 x 10^6 CFU of *Ft.* LVS and analyzed by flow cytometry for M1 macrophage polarization. **A**. M1 proinflammatory macrophage cell population as defined by F4/80 and macrosialin (CD68) expression and corresponding **B.** F4/80/CD68/IL-12^+ cells. Data is representative of n = 3-5 mice per group from 2 similar experiments. All P values were derived by one-way analysis of variance (ANOVA) using Tukey's post hoc analysis. * = $P < 0.05$ and ** = $P < 0.01$.

Figure 11

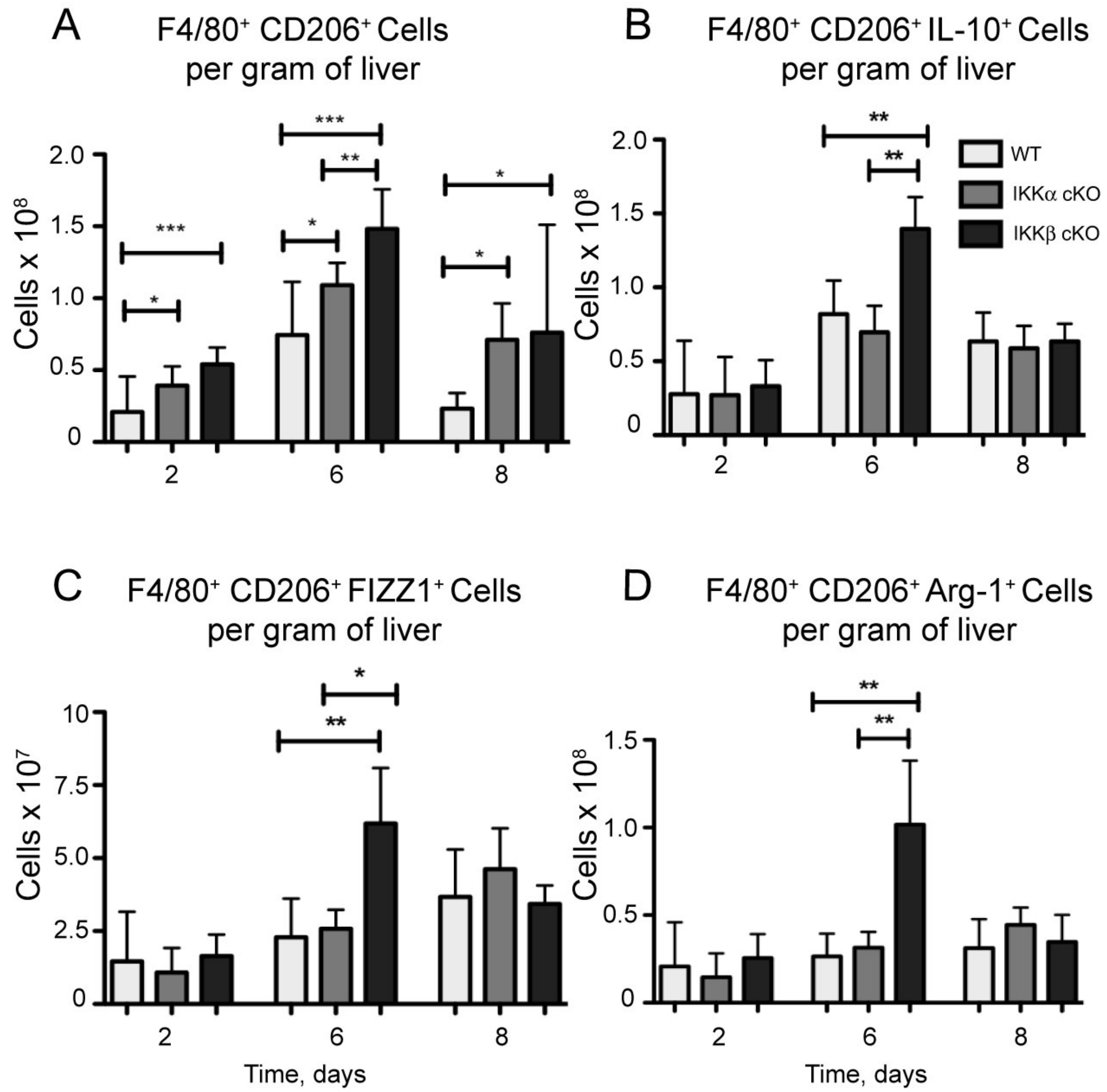


Figure 11: Anti inflammatory M2 macrophage activation in *Ft.* LVS infected IKKβ cKO mice. Hepatic NPCs were isolated from WT, IKKα and IKKβ cKO mice infected i.d. with 10^6 CFU i.d. of *Ft.* LVS and analyzed by flow cytometry for M2a macrophage specific markers. **A**. M2 anti-inflammatory macrophages identified by their F4/80 and mannose receptor (CD206) expression. **B-D** Intracellular staining of hepatic macrophages for additional M2 specific activation/polarization markers: **B**. F4/80/ CD206/IL-10. **C**. F4/80/CD206/Fizz1. **D**. F4/80/CD206/Arg-1. Data is representative of n = 3-5 mice per group from 2 similar experiments. All P values were derived by one-way analysis of variance (ANOVA) using Tukey's post hoc analysis. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

Temporal influx of neutrophils in the liver in IKKα and IKKβ cKO mice after intradermal *Ft.* LVS infection:

Neutrophils are early responders in the innate immune reactions toward bacterial infections. The cytotoxic effect of neutrophils can cause tissue damage to surrounding tissue if cells do not undergo a proper apoptotic program. LysM Cre expression mediates deletion of the IKKs in neutrophils as well, therefore, I questioned whether either of the IKKs in neutrophils could be involved in these aberrant responses to *Ft.* LVS infection seen thus far. Neutrophils were detected by flow cytometery from isolated liver NPCs according to their positive LY6B.2 7/4 (a pan neutrophil marker) antibody staining.

As shown in Figure 12, at two days post infection, IKKβ cKO mice exhibited a greater influx of neutrophils as compared to WT and IKKα cKO mice (3.05 and 5.55 fold, respectively). This means that the increase in bacterial burden (Figure 9A) seen in IKKβ cKO mice is not due to a loss of neutrophil migration. The increase in neutrophils also coincides with increased M1 macrophages (Figure 10A-B) at this early time point in infection. This suggests that IKKβ suppresses neutrophil infiltration at early time points.

Surprisingly, on day 8 post infection, both IKKα and IKKβ cKO livers showed small but significant increases in neutrophil recruitment to infected livers (1.98 and 1.47 fold, respectively to WT mice, and a 1.34 increase in IKKα cKO relative to IKKβ cKO).

Figure 12

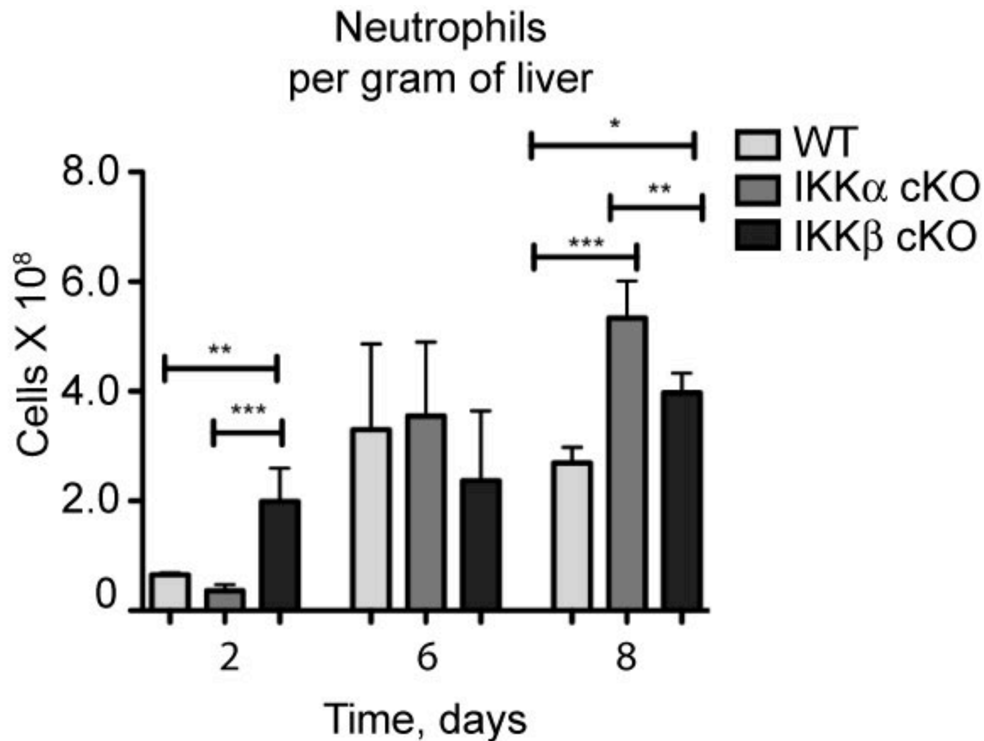


Figure 12: IKK deficiency results in a temporal influx of neutrophils in the Ft. LVS infected liver Hepatic neutrophils from animals injected with 10^6 *Ft*. LVS i.d. were analyzed by flow cytometry. Data is representative of 4 mice per group. All P values were derived by one-way analysis of variance (ANOVA) using Tukey's post hoc analysis. * = P < 0.05, ** = P < 0.01, *** = P < 0.001

IKKβ cKO mice present protracted IFN-γ expression in CD8 T cells

I also the considered the collateral effects of the loss of each IKK in macrophages and neutrophils may have on the natural killer (NK), T helper and cytotoxic cell populations as well as their ability to induce IFN-γ. As discussed, IFN-γ is considered a critical cytokine in granuloma formation, induction of inflammation, macrophage polarization and for the control of bacterial colonization.

Again, using flow cytometry from isolated liver NPCs, the NK cell compartment, defined as $NK1.1^+$ $CD3^-$ cells, were unaffected by loss of IKKβ, both in population size (Figure 13A) and in IFN-γ positivity (Figure 13B). A small, but significant change in IFN-γ positive cells were seen at day 6 post-infection in IKKα cKO mice, but this was less than a two-fold increase (Figure 13B. Fold change = 1.40). On day 8 post-infection, IKKα cKO infected livers had an 8.68 fold increase in the NK population (Figure 13A) but this did not correspond to an increase in IFN-γ positive cells at the same time point.

The T cell subsets, were defined either by their $CD4^+$ $CD3^+$ or their $CD8^+$ $CD3^+$ expression, to represent the T helper and cytotoxic T cell fractions, respectively. There was little change in the defining populations, except on day 8, where IKKβ cKO mice had small, but significant increases in both subsets of T cells with respect to WT animals (Figures 13C & E. 2.38 and 2.64 fold increase, respectively). Yet, this did not correlate with an increase in IFN-γ cells within the $CD4^+$ $CD3^+$ T cell fraction (Figure 13D). However, at all time points tested, IKKβ cKO mice showed increased IFN-γ positive $CD8^+$ $CD3^+$ cell populations (Figure 13F. Fold increases: day 2 = 8.04; day 6 = 2.19; day 8 = 7.29 with respect to WT mice and 7.70 fold increase on day 2 with respect to IKKα cKO mice).

Figure 13

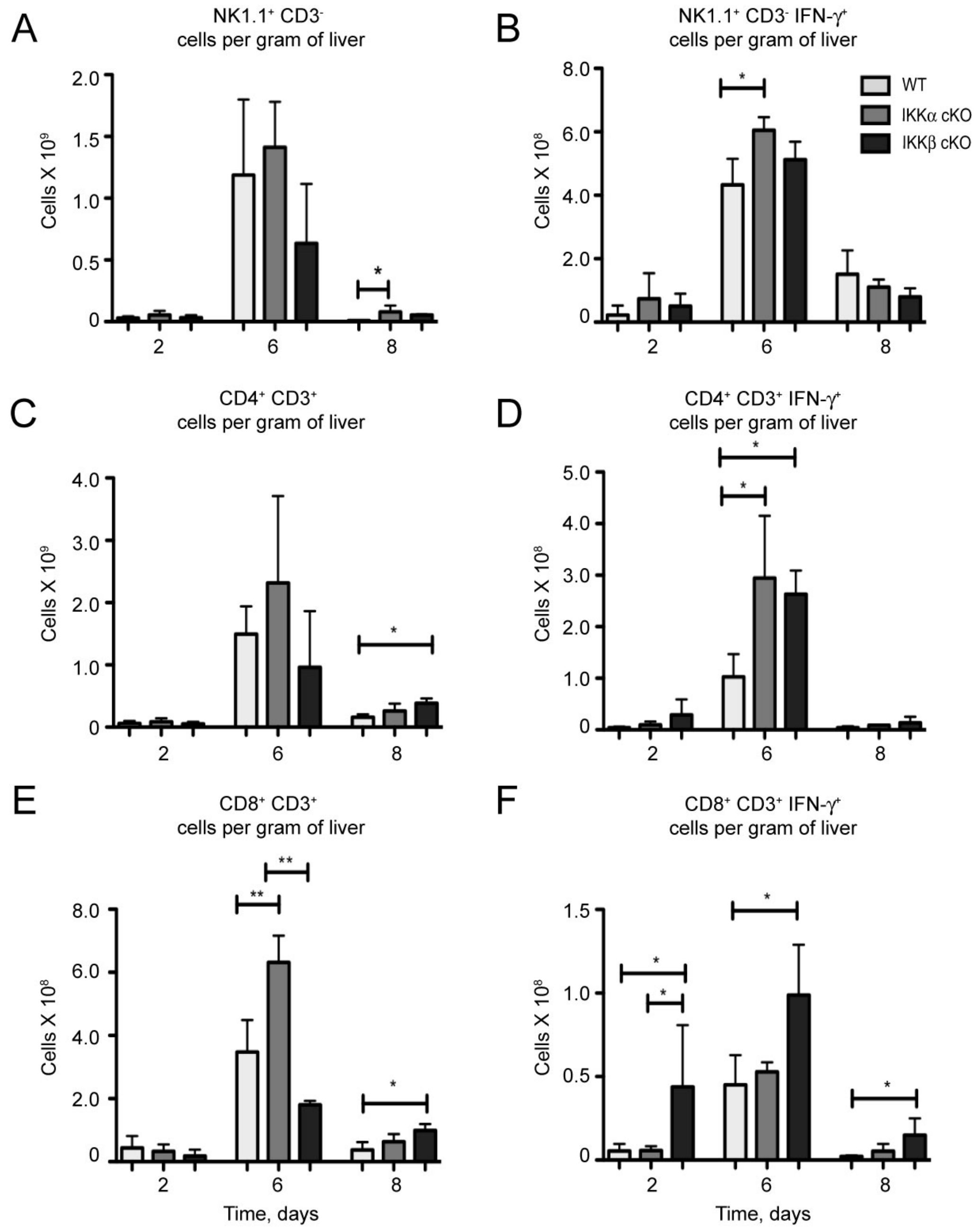


Figure 13: IKK cKOs have limited effects on the hepatic lymphocyte compartment in *Ft.* LVS infected mice. **A.** Natural killer (NK), **B**. $CD4^{+}CD3^{+}$ and **C.** $CD8^{+}CD3^{+}$ T-lymphocytes and their IFN-γ expression, **D-F** respectively, were analyzed by flow cytometry in *Ft*. LVS infected WT, IKKα cKO and IKKβ cKO mice. P values were derived by one-way analysis of variance (ANOVA) using Tukey's post hoc analysis. * = $P < 0.05$, ** = $P < 0.01$.

Infiltration of MDSCs in IKKβ cKO livers

Myeloid Derived Suppressor Cells (MDSC) have been recently described in *Francisella* containing hepatic lesions. Increases in these cells are seen during acute infection with certain bacteria and can drive T cell suppression via L-arginine metabolism. Decreased L-arginine levels causes down modulation of T cell function. This is results from the shortened half-life of CD3-ζ chain mRNA of the T cell receptor (TCR) (194). A functional TCR is required for regulation of T_H1 and T_H2 cytokine as well as CD8 T cell responses as CD3 is a co stimulatory molecule for both MHC I and MHC II dependent T cell recognition. To this end, I asked whether or not the deletion of macrophage and neutrophil IKKα or IKKβ affected the influx of this cell type. I again used a flow cytometry time course to look at the relative influx. Increases were seen in IKKβ cKO mice at day 2 post-infection compared to WT and IKKα cKO mice (Figure 14, IKKβ vs. WT fold change = 2.94, IKKβ cKO vs. IKKα cKO fold change = 5.73). The increased inflammatory phenotype of IKKβ cKO mice at this early time point with this concomitant increase in MDSCs is likely a host response enacted to suppress damaging inflammation. Fewer MDSCs were seen in IKKα cKO mice at the same time point and this agrees with their correspondingly lower bacterial burden and normal M1 macrophage profile. However, on day 8, IKKα cKO mice had an increased influx of MDSCs as compared to WT animals (Figure 14, fold change = 3.18). Since the inflammatory profile of IKKα cKO mice was similar to that of WT animals, this influx of MDSC may represent a source of new progenitors to repair the large granuloma cell death observed in IKKα cKO mice.

Figure 14

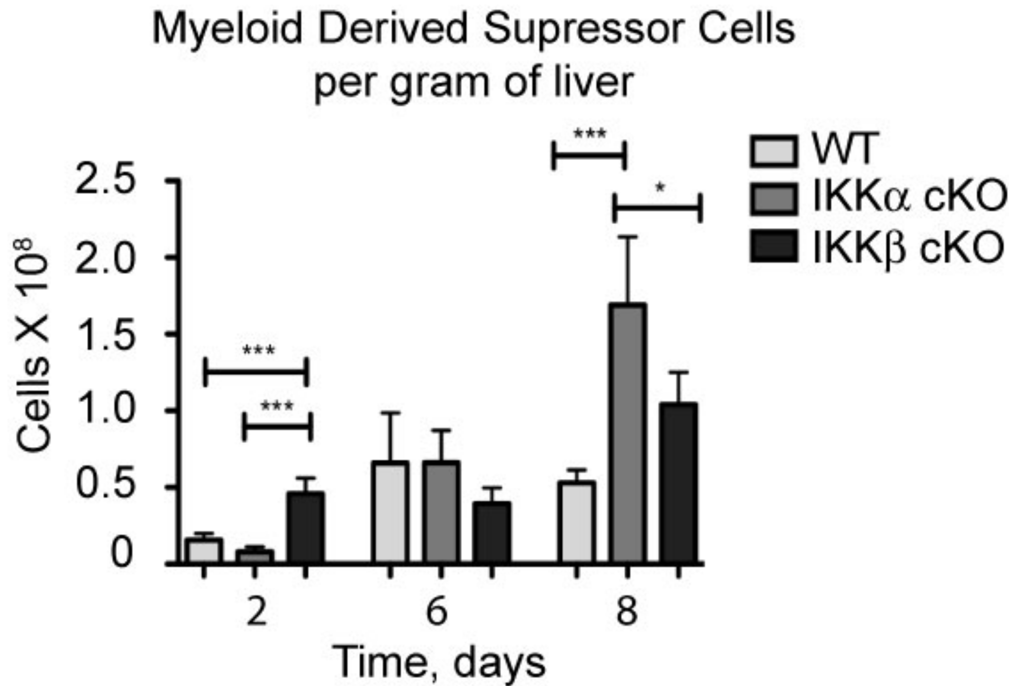


Figure 14: Differential requirements for myeloid derived suppressor cells in IKKα and IKKβ cKO mice. Hepatic myeloid derived suppressor cells $Gr1^+$ $CD11b^+$ were analyzed by flow cytometry after i.d. infection with 10^6 *Ft.* LVS in WT, IKKα cKO and IKKβ cKO mice. P values were derived by one-way analysis of variance (ANOVA) using Tukey's post hoc analysis. * = P < 0.05, *** = P < 0.001 with N= 4 mice per group

Chapter 6
Discussion

One long held tenet in NF-κB signaling is that IKKβ kinase activity is the key positive modulator of proinflammatory gene induction in the canonical NF-κB pathway. Alternatively, while a few gene specific roles for IKKα in canonical NF-κB signaling have been described, it is best understood for its kinase activity in regulating secondary lymphoid organ development in the non-canonical NF-κB pathway. The recent work by Fong (52) and Lawrence (51) showed that both IKKα and IKKβ are more dynamic than previously thought. *In vivo*, both kinases are capable of preventing inflammation by different mechanisms. The results of my work further expand the current knowledge IKK function in host immune response towards bacterial infection. In an infection model with *Francisella tularensis* LVS, I found that IKKα and IKKβ have distinct roles in survival, granuloma maintenance, bacterial colonization, as well as discrete effects on a number of different cell types involved in the host immune response.

Overall, the effects of an IKKα conditional knock out were less detrimental than a loss of IKKβ in this experimental model system. The main results for IKKα in the present study are: 1. IKKα expression in macrophages and neutrophils is not required for host survival after a lethal intradermal *Ft*. LVS infection, 2. IKKα is involved in the maintenance of granulomas by means of clearance of dead and dying cells in the liver after infection.

Roles for IKKβ cKO in this work are: 1. IKKβ is required for survival at high doses of bacteria, 2. IKKβ is required to control bacterial colonization in the liver, 3. IKKβ is required to maintain granuloma nucleation, 4. IKKβ is required to control inflammation at early time points of infection, 5. IKKβ is required to maintain inflammation at later time points Both IKKα and IKKβ also had extrinsic effects on other cell types important for cellular recruitment at specific time points during infection. A summary of my findings is listed in Table 8.

The results of IKK dependency in *Ft*. LVS infection differ from what was shown in the Lawrence and Fong studies in several ways. The first difference relates to the dependency of the IKKs in host survival to lethal infection. In my study, IKKα expression in macrophages and neutrophils was not required for survival, whereas IKKβ was necessary to protect mice against a high lethal dose of bacteria. In sharp contrast, the GBS model used in both the Lawrence and Fong studies showed that loss of IKKα kinase activity accelerated mortality while a loss of IKKβ was more resistant to lethal infection.

These contrasting results may be due to several reasons. First, there are differences between the bacteria used in each study. Like *Francisella*, GBS stimulates immune reactions through TLR2 and TLR6 (195). GBS is a Gram-positive extracellular bacterium. GBS does not contain LPS and therefore, does not signal through TLR4. *Francisella*, on the other hand, is a Gram-negative bacteria and its outer membrane contains LPS. However, the unusual structure of *Francisella* LPS exhibits low cytotoxicity and little TLR4 activation. Nevertheless, different bacteria activate immune responses in different ways. For example, *in vitro* infection studies have shown that extracellular bacteria such as *Staphylocccus aures* or *E. Coli* have differential

requirements for the efficient inflammasome-mediated, caspase-1-dependent IL-1β processing (196). These extracellular bacteria require additional stimulation of the P2X7 receptor by ATP that results in potassium efflux in order to activate caspase-1. On the other hand, P2X7 receptor stimulation is dispensable for intracellular bacteria such as *Listeria* and *Salmonella spp*., whereby inflammasome activation is dependent solely on bacterial PAMPS such as flagellin.

In addition, the inoculation routes differed in these studies. Lawrence and Fong utilized both intranasal (i.n.) and intraperitoneal (i.p.) methods of inoculation, whereas my study focused solely on intradermal infection. *Francisella* infection models utilizing the i.n. and i.p. modes of delivery are more severe as compared to dermal routes infections such as i.d. or subcutaneous (s.c.). Intradermal infection with *Ft*. is not usually lethal and mice tolerate inoculation doses that are several orders of magnitude higher than either the i.n. or i.p routes (63,197-199). Thus, intradermal *Ft.* LVS infection provides a good readout to analyze essential elements involved in host survival. To date, only a few molecular components have been found to be important for host survival through dermal routes of *Ft.* infection, including: MyD88, the molecular adaptor molecule for TLR signaling (150), the cytokines TNF-α and IFN-γ (106,200,201) in an i.d. infection with *Ft.* LVS, as well as the ASC, Caspase-1 (106) and AIM2 (124) components of the inflammasome in a subcutaneous infection with *Ft*. *novicida*.

An additional point relates to differences of host reactions in liver and lung tissue. Differences in liver and lung reactions are seen in *Ft*. LVS infections. The liver responds mainly by producing proinflammatory cytokines such as TNF-α, MCP1, KC/CXCL1 and IFN-γ in both i.p and i.d. infection, albeit to a greater extent in the i.p. route of infection.

Interestingly, the same infection differs in lung tissue responses, which appear to be anti-inflammatory producing little to none of these cytokines, but instead respond with increased IL-10 production (198).

In addition to differences in bacterial strain and route of inoculation, one final consideration surrounds the mouse model used by the Lawrence study to examine the role of IKKα in host survival. This infection model utilized a chimeric mouse model in which sublethally-irradiated WT mice were reconstituted with bone marrow from IKKα SS/AA transgenic mice. This strain contains mutations in two serines, 177 and 181, which are replaced by alanine residues, in the phosphorylation domain of IKKα. Importantly, although mutated, this model contains the IKKα protein and this protein still harbors activity independent of its phosphorylation domain. In fact, the study in which this transgenic mouse was first described (202), shows that the SS/AA mutation rescues embryonic defects in bone and skin morphogenesis seen in full knock out mice (36,37), but is defective in mammary gland development due to IKKα regulation of cyclin-D1 through RANKL (Receptor activator of NF-κB ligand) expression.

In spite of these differences, the results of my study show that IKKα in macrophages and neutrophils is not a contributing factor to host survival whereas IKKβ functions to protect animals from lethal infection of *Ft*. LVS at high doses. Moreover, this functional role of IKKβ implicates a direct role for macrophages and/or neutrophils in survival.

I also found novel roles for each IKK in the development and maintenance of hepatic granulomas in response to sublethal tularemia infection. In comparison to WT mice, fewer hepatic lesions developed in IKKα cKO infected mice. Although some

IKKα cKO granulomas became extremely large and usually contained central necrotic cores, overall they were well-contained and the remaining liver tissue appeared healthier and less inflamed in comparison to *Ft.* infected WT and IKKβ cKO mice. Despite the larger appearance and associated necrosis, a 90-day time course experiment showed that these unique granulomas in IKKα cKO mice had no obvious adverse effects on the host. Additionally, the defect in regulating granuloma size was not due to excess liver burden. The necrosis that develops within these large granulomas of IKKα cKO infected mice may be due to the host's ability to clear dead and dying cells. This would implicate a role for the professional phagocytes, macrophages and neutrophils and dendritic cells. Previously, neutrophils had been shown to be important in the clearance of dissolving hepatocytes from *Ft*. infected livers (68). IKKα cKO infected mice showed relatively normal influx of neutrophils ruling out defects in migration of these cells. Neutrophil effector functions, such as phagocytic ability, kinetics of uptake or cytotoxic effects which could lead to increased cell death were not evaluated in the present study and this could be an interesting topic to revisit in future work.

In contrast, histological analysis of livers from IKKβ cKO infected mice showed them to be highly inflamed. These mice developed far more granuloma-like lesions, which were often small (fewer than 20 cells per granuloma) and dispersed throughout the entire parenchyma, suggesting that IKKβ has an important role in controlling *Ft.* LVS infection. Indeed, in addition to this inflamed hepatic phenotype, IKKβ cKO mice also had increased organ burdens. Taken together, these observations indicate that IKKβ plays an important role in the control of bacterial colonization and growth in the liver. This effect was not due to an increase in bacterial uptake by IKKβ cKO

macrophages as evidenced by intracellular *Ft*. LVS LPS staining by flow cytometry, which was comparable to WT mice. Rather, these results suggest that the inflammatory response mounted in these mice is inadequate, and could reflect a defect in the production of bacteriocidal effectors such as NO or reactive oxygen. Alternative to this hypothesis, the poor nucleation of granuloma structures in IKKβ cKO mice may also allow bacteria to quickly spread throughout the parenchyma to invade healthy tissue.

Information relating to the molecular determinants involved in *Ft*. induced granulomas is limiting. To date, only IFN-γ (81) has been shown to affect granuloma structure in *Ft*. infection. As previously discussed, IFN-γ is required to maintain proper granuloma size and structure, contain bacterial antigens, control bacterial replication and induce iNOS production (81,169). In the present study, neither IKKα nor IKKβ cKO mice exhibited a decrease in IFN-γ producing NK, CD4 or CD8 T cells. Since the IKK deletion only occurs in macrophages and neutrophils, NK, CD4 and CD8 T cells are considered wild type, and thus, it is assumed they have normal cytokine secretion. The fact that the IKKs are also involved in maintaining granuloma structure independent of IFN-γ activation is a novel finding.

Due to the fact that IKK cKO mice did not exhibit any decreases in IFN-γ producing cells, a question remains as to the nature of the granuloma defects. Studies in other infection models provide insight into the kinds of molecules that might be involved in hepatic granuloma development and maintenance. Infection with *Leishmania donovani* in IL-12 deficient C57BL/6 mice fail to develop a proper T_H1 response, present significantly higher liver burdens and poorly organized granulomatous lesions that increase in number as infection progresses (203). Infection with eggs from the

helminthic parasite *Schistosoma mansoni* requires ICAM-1 (intercellular cell adhesion molecule 1) expression by hepatic endothelial cells for granuloma formation and this is induced by localized TNF-α production (204). Hepatic granuloma formation after *Mycobacterium bovis* bacille Calmette-Guérin (BCG) infection requires $CD4^+$ T cells. In this model, CD40L and IFN-γ are necessary to control bacteria within the granuloma, but not to maintain structure. However, TNF-α deficient mice form very large and poorly structured granulomas in response to BCG infection (205). These examples reveal that different infection agents have different requirements to induce or maintain the granuloma structure. At the same time, it overwhelmingly points to the role of cytokines in granuloma formation and maintenance.

To characterize the defects in each type of granuloma and the increased bacterial burden in IKKβ cKO mice more fully, I investigated the effects of either IKKα or IKKβ loss in macrophages for their M1 and M2a polarization. Akin to the T_H1 and T_H2 paradigm, M1 and M2a macrophages differentially regulate the cytokine environment in response to infection. IKKβ was recently shown to have anti-inflammatory function by means of inhibition of M1 macrophage polarization in a lung infection model with GBS (52). Loss of IKKβ from myeloid cells (this study used an IKKβ cKO transgenic mouse analogous to the one generated for my study) resulted in increased M1 activation as evidenced by increased IL-12, IFN-γ and a failure to resolve neutrophil infiltration in the lung. Therefore, it could follow that the enhanced inflammation in the livers of *Ft*. infected IKKβ cKO mice may also be the result of increased M1 polarization.

Both IKKα and IKKβ cKO infected mice displayed increased cell numbers of CD68 (M1) and CD206 (M2) macrophages compared to WT animals throughout the

course of infection, in spite of their markedly different granuloma phenotypes. However, unlike IKKα cKO M1 macrophages, IKKβ cKO M1 cells were clearly activated by virtue of their enhanced IL-12 production at day 2 post-infection. Moreover, IKKβ cKO infected hosts also had elevations in their M2 polarization markers by day 6 in contrast to both WT and IKKα cKO animals. However, since IKKβ cKO mice also had a higher bacterial burden this is suggestive of the host's failed attempt to initiate a wound-healing program to overcome the exacerbated inflammatory state in the liver. On the basis of the more inflamed granuloma phenotype and defective pathogen clearance response of IKKβ cKO mice, it is possible that IKKβ in macrophages is important for multiple reasons to maintain liver homeostasis during *Ft.* LVS infection. In addition, the shift from M1 to M2a macrophages in IKKβ cKO infected livers suggests that the roles for IKKβ in polarization may be necessary at discrete time points during the course of infection with IKKβ in WT mice acting to suppress an *Ft*. LVS mediated shift towards M2a polarization at later time points. In contrast, the role of IKKα in this disease model is independent of alterations in M1 and M2 polarization.

IFN-γ, in addition to its role in granuloma maintenance during *Ft*. infection (81), is also a factor required for M1 polarization. Natural killer cells are a major source of IFN-γ production in response to *Ft*. LVS infection, but other cell types, including T cells, contribute to IFN-γ production as well (81,206). The most revealing changes affecting IFN-γ production occurred in IKKβ cKO mice by CD8 T cells. It follows from this result that macrophage and/or neutrophil IKKβ signaling in WT *Ft*. LVS infected mice may serve to suppress IFN-γ activation CD8 T cells. Furthermore, CD8 T cell activation lasted throughout the course of infection indicating a long-term effect on these cells.

Generally speaking, activation of T cells is usually described as indirect. Macrophages and dendritic cells provide NK activating cytokines that then, in turn, provide additional signals, most notably, IFN-γ, to activate certain T cell subsets. This has been described as the IL-12/IL-18 > NK > T-cell axis (171,207,208).

Yet, some studies imply there may be a direct activation of T cells by macrophages and neutrophils. *In vivo*, macrophages can directly activate naïve CD8 T cells, a function previously ascribed to dendritic cells (209). In this study, adoptive transfer of gp33 (glycoprotein 33) peptide pulsed macrophages results in proliferation of naïve T cells that have a TCR specific for this peptide. In addition to proliferation, macrophages facilitate CD8 T cell induction of IFN-γ and generation of long-lived memory CD8 T cells. Another study, using a murine poxvirus infection model, shows that macrophages are necessary to mount antigen specific CD8 T cell responses. The CD8 T cell response is important in that the absence of these cells results in 100% mortality of infected mice. In addition, NK cells were only partially responsible for this effect as mortality persisted when mice were depleted of NK activity and resulted in only a modest decrease in mean time to death. This suggests macrophages may directly coordinate CD8 T cell responses (210).

Neutrophils may also modulate CD8 T cell function, though it is not clear if this is direct or mediated through NK activation. In a myocarditis mouse model, neutrophils are required to maintain antigen specific CD8 T cells infiltrates to heart tissue and draining lymph nodes. In addition to the disappearance of CD8 T cells, neutrophil depletion results in a chronic influx of CD4 T cells, protection from CD8 T cell induced damage and better survival (211). Another study involving photodynamic therapy (PDT) also

shows a role for neutrophils in CD8 T cell activation. PDT is a cancer treatment that uses light waves to induce immune reactions against tumors. Application of PDT induces neutrophil, primary CD8 T cell and memory CD8 T cell influx into tumors. Depletion of neutrophils results in the inability of the host to induce a strong CD8 T cell response (212). Given the results of these studies, macrophages and neutrophils appear to have positive effects on CD8 T cell activation.

Interestingly, IKKβ cKO mice showed concomitant increases in M1 macrophages and infiltrating neutrophils at early time points and sustained CD8 T cell IFN-γ production. This early state of hyperinflammation may result in long term CD8 T cell activation, even though the IKKβ cKO hosts appeared to compensate for this with increases in suppressive MDSCs and anti-inflammatory M2a macrophage polarization. Nevertheless, these proinflammatory events were unproductive as IKKβ cKO mice were unable to control bacterial colonization and growth. It remains to be seen if these effects are direct (macrophage or neutrophil mediated) or indirect (NK cell mediated), but would be an interesting topic for future work.

Macrophages and neutrophils have many effector functions that can be correlated to NF-κB induction. Gene transcription to promote inflammation in canonical NF-κB signaling involves not only the induction of inflammatory genes, but the activation of genes to control the duration and timing of activation. A classic example of this is a negative feedback loop that exists within the NF-κB pathway itself. Shortly after stimulation, the NF-κB inhibitor IκBa is upregulated, thereby limiting and dampening canonical NF-κB activation. Despite the obvious loss of IKKβ dependent proinflammatory gene induction in IKKβ cKO mice, inflammation can and does occur.

Although not well defined, proinflammatory activation of the ERK (extracellular signal-related kinase), p38, JNK (c-Jun N-terminal kinase) (152,213), PI3K/AKT (Phosphatidylinositol 3-Kinase) and SHIP (SH2 domain-containing Inositol 5-Phosphatase) (213) pathways have been reported in infections with *Francisella* spp. Due to the wide array of target genes activated by NF-κB, cross regulation of these pathways is likely to occur. Thus, some of the defects exhibited by IKKβ cKO mice may result from a loss of IKKβ-mediated cross regulation of other signaling pathways.

The limited effects of IKKα loss in this study were mainly correlated with granuloma maintenance in which where there was evidence of a loss of control in the clearance of dead and dying cells. Fewer defects were found in the regulation of other infiltrating cell types. The previously described role in apoptotic clearance by neutrophils may implicate this is the cell type contributing to granuloma defects in *Ft.* LVS infected IKKa cKO mice.

IKKβ loss resulted in multiple inflammatory defects, especially at early time points in infection. This implies that early in infection, IKKβ serves to limit inflammation of M1 macrophages and neutrophils, a result similar to that in the GBS infection model by Fong *et al*. (52). However, unlike the latter study, this loss of IKKβ-mediated control extended to increased bacterial colonization, protracted IFN-γ production by CD8 T cells and a clear but insufficient M2a polarization at day 6. Thus, my study also reveals that the requirements for IKKα and IKKβ in macrophages and neutrophils are pathogen specific and time dependent.

Table 8: Summary of results

Function:	IKKα:	IKKβ:
Survival	Not required.	Required at high doses of inoculum.
Granuloma	Regulates granuloma size and/or clearance of dead cells.	Regulates nucleation of the granuloma.
Colonization	Not required.	Required to control bacterial colonization and growth.
Macrophage uptake	Not required.	Not required.
M1 polarization	Not required.	IKKβ is anti-inflammatory at day 2.
M2 polarization	Not required.	IKKβ is proinflammatory at day 6.
Neutrophils	Not required for migration. Potential role in apoptotic clearance.	Inhibits migration at early time points.
NK Cells	Prevents NK influx or survival, but not required for IFN-γ induction at day 6.	Not required for influx or survival. Not required for IFN-γ induction.
$CD4^+CD3^+$ T cells	Not required for influx. Inhibits T cell IFN-γ induction at day 6.	Required for influx at day 8, but not for IFN-γ induction. Inhibits T cell IFN-γ induction at day 6.
$CD8^+CD3^+$ T cells	Prevents T cell expansion or influx at day 6. Not required for IFN-γ induction.	Prevents T cell expansion or influx at day 8. Protracts IFN-γ induction at all time points tested.
MDSC	Required for MDSC expansion or influx at day 8.	Required for MDSC expansion or influx at day 2.

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