IKKβ in Myeloid Cells Controls the Host Response to Lethal and Sublethal Francisella tularensis LVS Infection

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IKKβ in Myeloid Cells Controls the Host Response to Lethal and Sublethal *Francisella tularensis* LVS Infection

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Abstract

**Background:** The NF-κB activating kinases, IKKα and IKKβ, are key regulators of inflammation and immunity in response to infection by a variety of pathogens. Both IKKα and IKKβ have been reported to modulate either pro- or anti-inflammatory programs, which may be specific to the infectious organism or the target tissue. Here, we analyzed the requirements for the IKKs in myeloid cells *in vivo* in response to *Francisella tularensis* Live Vaccine Strain (FT. LVS) infection.

**Methods and Principal Findings:** In contrast to prior reports in which conditional deletion of IKKβ in the myeloid lineage promoted survival and conferred resistance to an in vivo group B streptococcus infection, we show that mice with a comparable conditional deletion (IKKβ cKO) succumb more rapidly to lethal *Ft. LVS* infection and are unable to control bacterial growth at sublethal doses. Flow cytometry analysis of hepatic non-parenchymal cells from infected mice reveals that IKKβ inhibits M1 classical macrophage activation two days post infection, which has the collateral effect of suppressing IFN-γ+CD8+ T cells. Despite this early enhanced inflammation, IKKβ cKO mice are unable to control infection; and this coincides with a shift toward M2a polarized macrophages. In comparison, we find that myeloid IKKα is dispensable for survival and bacterial control. However, both IKKα and IKKβ have effects on hepatic granuloma development. IKKα cKO mice develop fewer, but well-contained granulomas that accumulate excess necrotic cells after 9 days of infection; while IKKβ cKO mice develop numerous micro-granulomas that are less well contained.

**Conclusions:** Taken together our findings reveal that unlike IKKα, IKKβ has multiple, contrasting roles in this bacterial infection model by acting in an anti-inflammatory capacity at early times towards sublethal *Ft. LVS* infection; but in spite of this, macrophage IKKβ is also a critical effector for host survival and efficient pathogen clearance.

Introduction

NF-κB is an important signaling pathway for the induction and regulation of innate and adaptive immune responses toward bacterial infection. Microbial components and pro-inflammatory stress-like signals universally impact the activation of NF-κB transcription factors via the inhibitor of NF-κB kinase (IKK) signalosome complex. The signalosome contains two catalytic kinases, IKKα and IKKβ, and a third docking/regulatory subunit, IKKγ/NEMO [1]. The catalytic IKKs mediate the phosphorylation and subsequent degradation of the IkB family of cytoplasmic inhibitory proteins. IkB degradation liberates NF-κB transcription factors, resulting in nuclear translocation and target gene activation (for reviews see [2,3,4]). Canonical NF-κB activation requires IKKβ and IKKγ for IkB degradation, while the role of IKKα in the canonical signalosome is less clear. In addition, IKKα and IKKβ have also been shown to possess a variety of NF-κB-independent functions by regulating effectors of cell cycling, apoptosis, specific cellular differentiation pathways, chromatin activity and inflammatory responses [reviewed in. [5,6,7,8]].

In addition to the well-established roles the IKKs have on the induction of inflammation and adaptive immune responses, myeloid IKKα and IKKβ also limit inflammation in response to the extracellular bacterium Group B Streptococcus (GBS) or by *E. coli* LPS- (lipopolysaccharide) induced septic shock [9,10,11,12]. This unexpected behavior of the IKKs in myeloid cells led us to further investigate their anti-inflammatory properties in the context of the intracellular bacterium *Francisella tularensis* (*Ft.*).
Francisella tularensis is a highly infectious, Gram-negative, intracellular bacterium and is the causative agent of tularemia. Due to the high virulence of the human pathogenic strain ShuS4, the attenuated Live Vaccine Strain (F. LVS) is commonly used in mouse models of infection due to its ability to approximate human disease [13,14]. F. infects several cell types [15,16,17,18,19,20], but macrophages are considered the primary target of early infection [19,21,22]. Bacteria disseminate to colonize the lungs, liver, and spleen presumably through the hematogenous route [13,23]. Examination of F. LVS or ShuS4 infected organs indicates that lung, liver, and spleen each contribute distinct immune reactions [15,24,25,26]. Within 72 hours of intradermal (i.d.) or intraperitoneal (i.p.) inoculation of F. LVS, a pro-inflammatory gene expression profile is evident in liver and to a lesser extent in spleen, while lungs respond in an anti-inflammatory manner [24].

The virulence of Francisella has been attributed to its ability to down-modulate and/or evade host defenses. For example, outside the cell, Francisella are opsonized with complement proteins but are able to resist killing by complement-mediated lysis [27,28,29]. Opsonized bacteria are taken up readily by phagocytic cells, but avoid degradation by preventing fusion of host phagosomes to lysosomes and subsequently escape to the cytosol where they replicate [30,31,32,33]. Although F. is a Gram-negative bacterium, it has evolved an unusual LPS structure, which only minimally activates the pattern recognition receptor TLR4 (Toll-like Receptor-4) [34,35,36], and instead, innate immune recognition of F. LVS is mediated through TLR2 (Toll-like receptor-2) [37,38,39,40].

Francisella also interferes with anti-microbial defenses and inflammatory signaling pathways in several cell types, including macrophages and neutrophils. For example, F. LVS can prevent anti-microbial ROS (reactive oxygen species) production and NADPH oxidase assembly in human neutrophils [18]. F. LVS actively down-modulates TNF-α and IL-1 in the J774A murine macrophage cell line; this coincides with the inhibition of IkBα degradation and cross-tolerization to E. coli LPS (a TLR4 agonist) resulting in interference of NF-kB signaling [41,42].

There is also evidence that F. LVS affects macrophage polarization [43]. As early as 24 hours post infection, infected peritoneal elicited macrophages show properties of anti-inflammatory, alternatively-activated/M2 polarization resulting in increased expression of the M2 markers: mannose receptor (CD206), Fizz-1, Arg-1 and Ym1 [43]. Similar to the T cell polarization paradigm, macrophage polarization also depends on cytokine cues induced by the local environment (reviewed in [44,45]). In agreement with this, Francisella interferes with IFN-γ signaling, a key cytokine involved in macrophage polarization and bacterial control. F. LVS and its closely related subspecies F. novicida modulate IFN-γ signaling by suppressing tyrosine phosphorylation of the STAT1 transcription factor (Signal Transducer and Activator of Transcription 1) in human and murine mononuclear cells and this correlates with up-regulation of the STAT1 inhibitor SOCS3 (suppressor of cytokine signaling) [46].

Since infection with F. suppresses key inflammatory regulators, we hypothesized that the anti-inflammatory properties of myeloid IKK may compound and/or contribute to the progression of tularemia in vivo. To investigate this, we used myeloid-specific conditional knockout mice (IKK cKO) [47] to assess the roles of the IKKs in an intradermal (i.d.) model of F. LVS infection. We chose the liver as the model organ for our study because during tularemia infection, it supports pro-inflammatory changes [24], is colonized early in infection and has an abundant source of macrophages [48].

We found that myeloid IKKβ is required for survival during septic challenge, while IKKα cKO mice displayed mortality rates comparable to control mice. In a sublethal model of infection, flow cytometry analysis of hepatic non-parenchymal cells showed that loss of myeloid IKKβ, but not IKKα, results in polarization toward M1 macrophages in the liver early in the course of infection. Interestingly we found these effects are transient, as by mid-infection, macrophages polarize towards the M2a lineage.

Loss of myeloid IKKβ also induces protracted elevations in IFN-γ expressing CD8+ T cells, which persist throughout the course of infection. Despite these changes, IKKβ cKO mice are defective in control of bacterial growth. Finally, histological analysis shows that infection results in abnormal granulomatous liver responses in both strains of IKK cKO mice.

**Methods**

**Animals**

Myeloid specific ikk deletions were generated by crossing IKKα or IKKβ floxed mice with the LysM Cre expressing mouse strain [49] to generate IKKα flox/flox-LysM Cre or IKKβ flox/flox-LysM Cre strains, all of which were maintained on a C57BL/6 genetic background, as previously described [47]. To simplify nomenclature used throughout this text, mice are herein referred to as IKKα/β to represent control IKK floxed strains without myeloid deletion and IKKα cKO or IKKβ cKO to represent corresponding myeloid ikk conditional deletions. Mice were housed in a facility equipped with a 12:12 hour light:dark cycle in ventilated cages; and were fed a normal chow diet and autoclaved water ad libitum.

**Ethics Statement**

All procedures were performed in strict accordance with State University of New York at Stony Brook IACUC approved protocol ( Permit Number 0163).

**Bacteria**

F. LVS (ATCC 29684, American Type Culture Collection, Manassas, VA) were a kind gift from the laboratory of Dr. Martha Furie, and were grown according to published protocols [50]. Bacteria were used at OD600 between 0.2–0.4.

**Infection Model**

Bacteria were serially diluted to the indicated doses in sterile PBS in a final volume of 100 μl. Eight- to twenty-week old female mice were shaved and injected intradermally (i.d.) at the base of the tail. Mice were monitored for signs of distress (hunched posture, ruffled fur, loss of appetite, etc.). At lethal doses, these symptoms sometimes did not appear until hours before succumbing to infection. In addition, occasionally mice that appeared ill had spontaneously recovered. Sublethal infection did not induce symptoms of illness. Doses, listed as colony forming units (CFU), were confirmed by a retroplate colony count assay as described below.

**Survival Analysis**

The 50% Lethal Dose (LD50), was determined using the ICKαβ/β parental strain. Mice were infected i.d. with 106, 107 or 108 CFU of F. LVS and monitored daily for survival.
For comparison of survival between all strains, mice (10–15 per group) were injected with an LD50 dose 10^8 CFU i.d. and monitored twice daily over a period of 14 days. Kaplan-Meier survival curves were generated and relative mortalities in response to lethal infection were compared by Mantel-Cox log-rank test.

**Organ Burden Determination and Retroplate Colony Counts**

Organ burden was determined using limiting-dilution culture. Briefly, sections of the median lobe (~0.1–0.2 g) of infected livers were aseptically removed and placed in a pre-weighed sterile tube containing 1 ml of sterile PBS. The remaining liver and section were weighed. The section was homogenized in a stomacher bag (Fisher Scientific, Pittsburgh, PA) and serially diluted. Neat homogenates and serial dilutions were plated and incubated for 3 days at 37°C. The resulting colonies were counted and represented as a concentration per gram of liver. This concentration was then multiplied by the weight of the total liver and corresponding dilution factor to derive the total organ burden. Retroplate colony counts were performed in a similar manner. First, serial dilutions on the inoculation dose were made. Dilutions were then plated and incubated as above. The resulting colonies were counted, multiplied to respective dilution factors and represented as CFU/ml. The limit of detection for these assays was 200 CFU.

**Histology**

Organs were fixed in 10% neutral buffered formalin (Sigma Diagnostics, St. Louis, MO) and embedded in paraffin, sectioned at 5 μm, rehydrated to water through graded alcohols. Tissues were stained with hematoxylin and eosin (H & E), dehydrated in graded alcohols, cleared with xylene and mounted with permanent mounting medium (EMS, Hatfield, PA). An experienced pathologist performed a blinded analysis on H & E sections to identify any abnormalities in lung, liver and spleen. Images were captured on an Olympus BX41 light microscope (Olympus, Tokyo, Japan).

Granuloma counts from 8 mice/group were quantified by examination of 10 fields/liver under 200× magnification. Poisson distribution was assumed and fitted to Poisson regression by taking the square root of count data and mean comparisons were made with one-way ANOVA and Tukey’s post-test. Data was then back-transformed into original units of granulomas/200× field (0.150 mm^2).

**Immunohistochemistry**

Tissues were fixed in neutral-buffered formalin for 48 hrs, embedded in paraffin and sectioned at 5 μm. Deparaffinization, rehydration and antigen retrieval was performed by incubating slides in Trilogy™ solution (Cell Marque, Rocklin, CA) for 1 hr at 90°C. Slides were rinsed in TBST (Tris Buffered Saline-Tween; Thermo-Scientific, Freemont, CA), blocked in goat alcohols, cleared with xylene and mounted with permanent mounting medium (EMS, Hatfield, PA). An experienced pathologist performed a blinded analysis on H & E sections to identify any abnormalities in lung, liver and spleen. Images were captured on an Olympus BX41 light microscope (Olympus, Tokyo, Japan).

Granuloma counts from 8 mice/group were quantified by examination of 10 fields/liver under 200× magnification. Poisson distribution was assumed and fitted to Poisson regression by taking the square root of count data and mean comparisons were made with one-way ANOVA and Tukey’s post-test. Data was then back-transformed into original units of granulomas/200× field (0.150 mm^2).

**Non-parenchymal Cell (NPC) Isolation**

NPC’s were isolated by the methods of Rasmussen et al [51]. Briefly, mice were injected with 10^6 CFU of Ft. LVS and euthanized at the indicated time points. On the day of harvest, livers were perfused with 1 mM Citrate (Sigma, St. Louis, MO) in Hanks Buffered Saline Solution (HBSS, Gibco/Invitrogen Carlsbad, CA). Livers were minced to 1 mm pieces and placed in digest buffer containing 0.05% Collagenase II, 0.002% DNase1 and 1% BSA (all from Sigma-Aldrich, St. Louis, MO) in HBSS, rotating at 75 RPM, 37°C for 45 minutes. Digests were triturated 10X and filtered through a 70 μm cell strainer (BD Falcon, San Jose, CA). Cells were pelleted and resuspended in ACK hypotonic buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA) for 5 minutes to lyse RBCs. Cells were washed in PBS and NPCs were isolated by flotation method using OptiPrep density gradient (AxisShield, Oslo, Norway), according to manufacturer protocol C-24. Upper and lower isolates were collected and the NPC fraction was confirmed by flow cytometry. The upper isolate was >97% CD45+ (eBioscience, San Diego, CA).

**Flow Cytometry**

NPCs isolated from infected livers were stained with antibodies for surface and/or intracellular markers for immunophenotyping and cytokine positivity according to manufacturer suggested protocols. Briefly, harvested cells were immediately incubated in growth media (RPMI 1640, 5% heat inactivated FBS [Invitrogen/Life Technologies, Carlsbad, CA] without antibiotics) containing 1 μg/ml GolgiPlug (BD Biosciences, San Jose, CA) for 6 hours to prevent cytokine release; no exogenous stimuli were added during
the incubation period. GolgiPlugs was added to all subsequent flow cytometry buffers until the fixation step. Cells were washed 1× in PBS and 1× in FACS Stain Buffer (FSB: 1% heat inactivated FBS, 0.09% w/v Na3 in PBS). Fcγ receptors were blocked with CD16/32 antibody (eBiosciences, San Diego, CA). Surface epitopes were stained with antibody for 30 min., washed 2 times with FSB. Cells were fixed and permeabilized using BD Cytofix/ Cytoperm buffer kit (BD Biosciences, San Jose, CA) for 20 min. Intracellular antibodies were added to cells and incubated for 30 min. Unlabeled antibodies required secondary labeling was carried out in a third staining step as above. All labeling and permeabilization steps took place in the dark with rotation. The following antibodies were used in this study: Arg1 (Everest Biotech, Oxfordshire, UK), PE-CD120a (B111922, BioLegend, San Diego, CA), FITC-CD3ε (145-2C11, eBiosciences, San Diego, CA), FITC-CD4 (RM4-5, BioLegend), FITC-CD8α (53-6.7, BioLegend), PE-CD11b (M1-70, BioLegend), PerCP-Cy5.5-CD11b (M1-70, BD Biosciences, San Jose, CA), FITC-CD45 (30.F11, BioLegend), APC-Fc-80 (BM8, eBiosciences), FITC-F4/80 (BM8, eBiosciences), RELMα (AbD Serotec, Raleigh, NC), PerCP-Cy5.5-NK1.1 (PK136, BD Biosciences), RELMβ (XMG1.2, BD Biosciences), APC-IL-10 (JES5-16E3, BD Biosciences), APC-IL-12p40/70 (XMG1.2, BioLegend), APC-F4/80 (BM8, eBiosciences), FITC-F4/80 (BM8, eBiosciences), PE-CD11b (M1-70, BioLegend), PerCP-Cy5.5-CD11b (M1-70, BD Biosciences, San Jose, CA), FITC-CD45 (30.F11, BioLegend), APC-Fc-80 (BM8, eBiosciences), FITC-F4/80 (BM8, eBiosciences), Ft. LVS (Rabbit polyclonal, a generous gift of Dr. Jorge Benach), APC-IFN-γ (XMG1.2, BD Biosciences), PE-IL-10 (JES5-16E3, BD Biosciences), APC-IL-12p40/70 (C15.6, BD Biosciences), FITC-pan-Neutrophils/Ly-6B.2 (7/4, AbD Serotec, Raleigh, NC), PerCP-Cy5.5-NK1.1 (PK136, BD Biosciences), RELMα/FIZZ1 (Rabbit Polyclonal, Abcam, Cambridge, MA), PE-Donkey anti rabbit IgG (Abcam), PE-Donkey anti goat IgG (Abcam). Acquisition was performed on FacsCalibur flow cytometer (BD Biosciences, San Jose, CA) and data was analyzed using FlowJo V9.0.1 (TreeStar, Ashland, OR). Cell counts are normalized as counts per gram of liver.

Statistical Analysis

Data were analyzed by one-way ANOVA with Tukey’s multiple comparison post-test using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA).

Results

Reduced Survival of Myeloid IKKβ-deficient Mice Following Lethal Intradermal Ft. LVS Challenge

The IKKs are important signaling factors involved in orchestrating host immune responses to a wide variety of pathogens, yet the requirements for IKK signaling in myeloid cells during Ft. LVS infection are unknown. To address this, we employed Cre/ Lox mediated recombination in which IKKα or IKKβ are conditionally deleted from the myeloid compartment of adult mice [47,49,52]. The severity of Francisella infection varies by bacterial strain, host genetic background, dose and route of administration [13,23,53]. In order to establish the correct dosage for our model system, we began with a pilot experiment in which we intradermally (i.d.) injected IKKβ KO mice at various doses of Ft. LVS in order to establish the median lethal dose (LD50). After retroplate assay correction, the LD50 was determined to be 107 CFU of Ft. LVS (Fig. S1 A-C).

We first investigated if either IKKα or IKKβ in myeloid cells were required for host survival to Ft. LVS infection. We injected IKKα KO and IKKβ KO mice with the LD50 dose of 107 CFU Ft. LVS and scored animals for their relative survival rates. All IKKβ KO mice succumbed to infection by day 10 with a comparative Log-rank test P value of 0.0014 (Fig. 1); there were no significant differences in the survival rates among IKKα KO and IKKα KO mice.
tissue (iBALT), which can be induced in humans and mice upon exposure to infection and inflammation and are considered tertiary sites of B and T cell priming reactions [54]. Immunohistochemical analysis to detect *F. LVS* antigen (Fig. S2) showed a limited infection within the lung parenchyma. The overall conclusion about lung reactions in all three strains of mice was that they were relatively mild. These findings are consistent with previous reports for intradermal infection, not only with *F. LVS* [55], but also with the more virulent strain *Francisella tularensis* Schu4 [23].

The spleen and liver undergo pronounced inflammatory reactions upon *Ft. LVS* infection and are characterized by the development of granuloma foci, which act as a microenvironment that serves to contain infection and clear debris from infected and dying cells [19,23,51,56]. Histological examination of the spleens from uninfected mice appeared normal and showed clearly demarcated red and white pulp. Follicles, germinal centers and the surrounding cuff of the marginal zone were well represented (data not shown). At two days post infection, the red pulp was marginally expanded, but other structures were qualitatively normal in all three strains. A few small granuloma-like lesions began to appear within the red pulp of most animals (Figs. 3A–C and Fig. S3). More severe histopathological changes to splenic architecture progressed through days 6 and 9 post-infection (data not shown).

Figure 2. Dissemination of *Ft. LVS* leads to lymphocytic aggregate development in IKKβ cKO mouse lung. H & E stained lung tissue was examined for histological changes at days 2 and 9 (A–F) after i.d. infection with 10⁶ CFU of *Ft. LVS*. (A) IKKα cKO and (B) IKKγ cKO mice show evolution and (D, E) resolution of inflammation, while (C and F) IKKβ cKO mice exhibit a delay in resolution concordant with the appearance of (F, inset) lymphocytic aggregates. Figure insets are taken from regions marked by rectangles. Yellow arrows indicate neutrophils, blue arrows indicate lymphocytic infiltrate, yellow arrowheads indicate lymphocytic aggregates. Scale bar = 500 μm, 20× magnification, inset scale bar = 50 μm, 400×magnification). Representative sections are shown from at least three independent experiments.

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not shown and Fig. 3D–F, respectively). The white pulp showed evidence of expansion and macrophage and lymphocytic infiltrates were seen in the red pulp. The number of granulomas marginally increased in IKK\(a\) and IKK\(\beta\) cKO mice. However, in IKK\(\beta\) cKO infected mice, the red pulp was often completely effaced with coalescing granulomas making accurate quantification difficult. Neutrophils were sometimes present within these granulomas (Fig. 3F, inset). Splenomegaly was apparent in all strains (Fig. 3G). Comparisons of spleen/total mouse weight ratios (spleen index) showed a \(-3\) fold change by day six. The spleen index modestly increased to \(3.5\)–\(4\) fold by day nine. No significant differences in spleen indices between strains were observed. Immunohistochemical analysis of Ft. antigen in spleen at \(2\) days post infection showed numerous bacterial foci (Fig. S3 A–C) in all three strains of mice. Bacteria were localized predominantly in the red pulp, but were occasionally observed in the white pulp as well (Fig. S3 A). By day 9, there was a marked decrease in the number of bacterial foci, indicating resolution of infection.

Granuloma formation in liver was evident at two days post-infection in all three strains (Fig. 4A–C). At day 2, granuloma counts in IKK\(\beta\) cKO mice were at least two fold greater in number than those of IKK\(a\) or IKK\(\alpha\) cKO mice (Fig. 4G, \(P<0.0001\)). By day 9, granuloma formation continued to increase in IKK\(\beta\) cKO at a rate far greater than either IKK\(a\) or IKK\(\alpha\) cKO mice. Additionally, the granuloma-like structures that developed in IKK\(\beta\) cKO infected mice were often extremely small and decondensed, resulting in livers replete with inflammatory cells (Fig. 4F). Some granulomas in IKK\(\alpha\) cKO mice developed into very large, macroscopically discernable structures that contained a central core filled with necrotic and cellular debris (Fig. 4E; note that panel 4E is a lower magnification than 4D and 4F), and although large, these structures were well contained by a cuff of mononuclear cells, epitheloid histiococytes and occasional lymphocytes. Although both spleen and liver showed increased numbers of granuloma foci in IKK\(\beta\) cKO mice, the liver also showed clear discernable defects in granuloma maintenance in both mutant strains of mice. Since the liver reacts with a strong pro-inflammatory response to Ft. LVS intradermal infection \[24\] and contains a rich source of macrophages, we focused the remainder of our experiments on the liver to further investigate the anti-inflammatory effects of myeloid IKK\(\beta\) during the course of tularemia.

**Myeloid IKK\(\beta\) is Required for Control of Bacterial Growth in the Ft. LVS Infected Liver**

Bacterial colonization is first detectable in Ft. infected livers at 2–3 days post infection \[19,24,51,56\]. In order to determine if the observed granuloma defects in the liver were due to increased bacterial colonization, a retroplate time course study to determine organ burden was performed on liver homogenates from mice infected with \(10^8\) CFU of Ft. LVS. At day 2 post infection, both IKK\(a\) and IKK\(\alpha\) cKO mice showed limited colonization of the liver while, even at this early time point, IKK\(\beta\) cKO mice presented with increased bacterial loads that persisted through day 14 (Fig. 5A).

Macrophages are considered a primary cellular target of Francisella infection \[22,57\] and are also a major component of Ft. induced granulomas \[51\]. Since macrophages are one of the cell types affected by our conditional knockouts, we next questioned whether the macrophages from these mice were comparably infected relative to control mice. To address this, non-parenchymal cells (NPCs) were isolated from the livers of infected mice, stained with F4/80 and CD11b antibodies followed by intracellular staining for Ft. LVS and then subjected to flow cytometry. Interestingly, IKK\(\beta\) cKO infected mice showed only modest increases in the number of Ft. LVS positive macrophages throughout the course of infection (Fig. 5B). To account for a possible loss of cells due to infection, we analyzed macrophage and neutrophil cell populations from each strain. We isolated hepatic NPCs from sublethally Ft. LVS infected livers and performed a flow cytometry analysis time course to evaluate the populations of F4/80\(^+\) CD11b\(^+\) expressing macrophages and Ly6B.2\(^+\) expressing neutrophils. Prior to infection (day 0), all three strains of mice yielded comparable numbers of macrophages and neutrophils (Fig. 6A & B, respectively). Modest increases were observed in the macrophage population but persisted throughout the 8-day time course (Fig. 6A). As early as two days post infection, increases in neutrophils were evident in IKK\(\beta\) cKO mice and by day 8, neutrophils were elevated in both mutant strains, relative to the parental strain (Fig. 6B). Taken together, these data suggest that the increased liver burden in IKK\(\beta\) cKO mice or granuloma maintenance were due to defects in myeloid function related to control of bacterial growth and spread of infection rather than overt initial increases in macrophage infection (Fig. 5B) or loss of cells from the myeloid compartment (Fig. 6A–B).

**Myeloid Function in the Granuloma**

To further examine myeloid function in liver granulomas, we performed immunohistochemical assays to detect the spatial localization of LVS antigen, production of the inflammatory mediator iNOS (inducible nitric oxide synthase) and induction of caspase-3 (CC-3) activation. Figure 7 shows representative granulomas from each of the three strains of mice. LVS antigen was largely restricted to the granuloma (Fig. 7, A–F), however, a small number of Ft. antigen positive cells (0–11 cells/200\(\times\) field) were sometimes found within the parenchyma. Ft. antigen positive cells were almost two-fold higher in IKK\(\beta\) cKO mice, but this correlated with higher bacterial burdens.

Lindgren et al \[58\] reported that intradermal inoculation of Ft. LVS in iNOS (inducible Nitric Oxide Synthase) deficient mice results in decreased host survival, increased bacterial colonization and increased liver damage concurrent with the appearance of numerous small granuloma-like foci in the liver. Moreover, iNOS, in addition to induction by IFN-\(\gamma\), is also a secondary response target gene activated through TLR signal transduction \[59,60\]. We next asked if iNOS was similarly expressed in liver granulomas of IKK deficient mice. At two days post infection, we observed iNOS positivity in 54%, 40% and 54% in hepatic granulomas from IKK\(a\), IKK\(\alpha\) cKO and IKK\(\beta\) cKO mice, respectively (Fig. 7 B, H, N). By nine days post-infection (Fig. 7 E, K, Q), only modest increases in the number of iNOS-positive granulomas (~10%) in IKK\(a\) and IKK\(\beta\) cKO infected mice were noted. In comparison, the number of IKK\(\alpha\) cKO iNOS positive granulomas remained essentially unchanged at 42%.

Rapid induction of apoptosis in bacterially infected cells is an immune defense mechanism that helps to limit the spread of infection. We analyzed induction of apoptosis in hepatic granulomas using the apoptotic marker, cleaved caspase-3 (CC-3). CC-3 staining was largely restricted to granulomas. At early time points in infection, ~35% of all granulomas scored positive CC-3 in all strains of mice. However, only a few (~1–8) positive cells were found per granuloma (Fig. 7M–R), and this was consistent between all strains of mice. By day nine, CC-3 positive granulomas from IKK\(a\) mice was reduced to 11.3%, while IKK\(\alpha\) and IKK\(\beta\) cKO mice retained an overall 25 and 22% CC3 positive granuloma score, respectively.
Figure 3. Exacerbated histopathology in the spleen in IKKβ cKO mice. Spleen sections were stained with H & E and evaluated for histopathological changes at days 2 and 9 post i.d. infection with 10^6 CFU of Ft. LVS. Granuloma foci developed in the red pulp of the spleen (yellow arrowheads) in (A) IKK^{ff}, (B) IKKα cKO and (C) IKKβ cKO mice as early as 2 days post-infection. (D–F) These foci increased in number by day 9. Inset in (F) is representative of a typical granuloma within the red pulp. The yellow arrow points to a neutrophil within the granuloma. (G) All strains of mice developed splenomegaly during the course of infection as determined by the spleen index (ratio of spleen weight to body weight x 100). Scale bar for panels A–F = 500 μm, 20× magnification, inset scale bar in F = 50 μm, 400× magnification. Representative sections are shown from at least three independent experiments.

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IKKβ deficiency in myeloid cells was previously reported to induce classical/M1 macrophage polarization in response to GBS infection [11], we, therefore, questioned whether this also occurs in the liver in response to *Ft.* LVS infection. To address this, mice were injected i.d. with 10^6 CFU of *Ft.* LVS and we analyzed the NPC fraction isolated from the livers of infected mice in a time course experiment using flow cytometry. To first broadly identify
M1 and M2a macrophage subpopulations, we scored macrophages for their F4/80 expression and either macrosialin (murine CD68) or the mannose receptor (CD206), (Fig. 8A and 9A, respectively). Next, we evaluated each subpopulation for expression of the prototypical M1 cytokine, IL-12 (Fig. 8B) or multiple M2 activation markers, including IL-10, arginase-1 and RELMα/FIZZ1 (Fig. 9B–D).

We observed a 5-fold increase (P = 0.0040) in the number of M1 macrophages (F4/80+CD68+IL-12+) in IKKβ cKO mice at 2 days post-infection (Fig. 8B). This data indicates that myeloid IKKβ contributes to the suppression of M1 classical activation in the liver in response to sublethal Ft. LVS infection. However, this IKKβ-mediated M1 suppression only occurred in a short time period early in infection, as by day 6 there was a shift toward M2a activation as indicated by increases in F4/80+CD206+ staining in coordination with several other M2a-associated markers (Fig. 9B–D) including: IL-10, a 2 fold increase P = 0.0019; FIZZ1, a 6 fold increase P = 0.0085; and Arg-1, a 9 fold increase P = 0.0041).

Livers of Ft. LVS Infected IKKβ cKO Mice Present Protracted Activation of CD8+ T cells in the Liver

We next evaluated the collateral effects of IKK myeloid deletion with respect to the activation of IFN-γ by other cell types after sublethal i.d. Ft. LVS infection. Again, using a flow cytometric approach on liver NPCs, we scored for IFN-γ activation in NK (NK1.1+CD3−), CD4+ and CD8+ T cell (CD4+CD3+ and CD8+Figure 5. IKKβ controls Ft. LVS growth in the liver. (A) Organ burdens were determined by retroplate assay from liver homogenates of mice injected i.d. with 10⁶ CFU Ft. LVS at days 2, 6, 9, and 14. Data is presented as CFU of Ft. LVS per gram of liver. Data were pooled from two independent experiments. The dashed line represents the assay limit of detection, 200 CFU. (n for IKKα+/+, IKKα cKO, IKKβ cKO on day 2 = 8; day 6 = 7; day 9 = 6; day 14 n = 6) (B) Liver macrophages were analyzed for infection by flow cytometry. Statistical analysis was performed by one-way ANOVA followed by Tukey’s ad hoc post-test. (n for IKKα+/+, IKKα cKO, IKKβ cKO on day 0:5/5/5; day 2:4/4/4; day 6:4/4/4; day 8:4/4/3, respectively). Bars represent the mean ± SD. **P < 0.01, ***P < 0.0005, ****P < 0.0001.

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Figure 6. Myeloid lineage response in Ft. LVS infected livers. We analyzed (A) macrophage and (B) neutrophil populations in the liver by flow cytometry during the course of sublethal Ft. LVS infection. Statistical analysis was performed by one-way ANOVA followed by Tukey’s ad hoc post-test. (n for IKKα+/+, IKKα cKO, IKKβ cKO on day 0:5/5/5; day 2:4/4/4; day 6:4/4/4; day 8:4/4/3, respectively). Results are representative of at least two independent experiments. Bars represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.0005, ****P < 0.0001.

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CD3+, respectively) populations. The NK cell populations from IKKa and IKKb cKO infected mice were largely similar to IKKf/f control animals (Fig. 10A). A small increase in NK cells were seen in IKKa cKO animals on day 8 post-infection, yet we did not observe corresponding increases in IFN-γ+ cells at the same time point (Fig. 10B). Increases in both CD4+ and CD8+ T cell populations were observed at later time points in infection in IKKb cKO mice (Fig. 10C and E). In CD8+ T cells, these increases correspondingly correlated to greater increases in the number of IFN-γ+ cells (Fig. 10E and F). Interestingly, increases in IFN-γ+ CD8+ T cells from IKKb cKO infected mice were protracted throughout the entire infection time course (Fig. 10F). This trend was not observed in IKKf/f or IKKα cKO infected mice. These results suggest that myeloid IKKβ may function in an extrinsic manner to suppress the influx of activated IFN-γ producing cells.

Discussion

The results of this work shed new light on the contributions of the IKKs in myeloid cells during Ft. LVS infection and also extend previous findings on the in vivo roles of the IKKs in myeloid cells in response to bacterial infections. In spite of the anti-inflammatory properties demonstrated by others [9,10,11], myeloid IKKs exhibit notable differences in response to the intracellular bacterium Ft. LVS. Here, we found that myeloid IKKβ has a protective role in response to lethal challenge. At sub-lethal doses, histopathology and retroplate culture assays showed that myeloid IKKβ helps regulate the granulomatous response and control infection.

We also examined the effect loss myeloid IKKβ had on macrophage polarization. Indeed, IKKβ inhibits classical M1
macrophage activation. However, this effect was limited to early time points of infection, in spite of a protracted elevation of IFN-\(\gamma\) and CD8\(^+\) T cells during the course of infection. On the contrary, we observed only limited roles for myeloid IKK\(\alpha\) during \(Fr.\) LVS infection. Loss of myeloid IKK\(\alpha\) resulted in similar survival rates compared to control mice and a reduction in bacterial colonization at early time points. The most notable difference in IKK\(\alpha\) cKO mice was in the appearance of large, necrotic, granuloma-like foci, which formed in the liver at late stages of infection, suggesting that IKK\(\alpha\) in myeloid cells may be involved in clearance of dying cells. These foci were cleared after 90 days of infection (data not shown) by a yet unknown mechanism.

IKK\(\beta\) but not IKK\(\alpha\) in Myeloid Cells Contributes to Host Survival to a Lethal Dose of \(Fr.\) LVS

The survival results we obtained in our study are in contrast to those reported for myeloid IKK deficient mice responding to a lethal Gram-positive bacterium GBS (Group B Streptococcus) challenge [10,11]. In this previous study, mice devoid of myeloid IKK\(\beta\) were more resistant to a lethal challenge with GBS [11]. Here, we found that intradermal inoculation with Gram-negative bacterium, \(Fr.\) LVS rendered myeloid IKK\(\beta\) deficient mice much more susceptible to lethal infection. These results correspondingly correlated to levels of bacterial titers, as there was increased bacterial clearance in the GBS model [11], while we observed increased colonization in the \(Fr.\) LVS model. In addition, loss of functional IKK\(\alpha\) (\(\text{Ikk}^{\alpha\text{AA/AA}}\) phosphorylation mutant) in the myeloid compartment resulted in increased susceptibility to GBS infection [10], while here, we found IKK\(\alpha\) cKO mice maintain a comparable susceptibility in response to \(Fr.\) LVS infection to control animals. These contrasting results in survival between the \textit{Francisella} and GBS infection models in myeloid IKK deficient mice suggest that there are differential requirements for downstream TLR effectors, such as the I KKs, during septic challenge. In fact, similar differences in survival patterns have been reported for the TLR adapter protein MyD88 (Myeloid-differentiation factor 88). Mice deficient in MyD88 are protected from GBS infection at high infection doses, [61], while \(Fr.\) LVS infected mice are extremely susceptible to MyD88 loss at low doses [55]. Moreover, increased mortality occurred in myeloid IKK\(\beta\) deficient mice at the LD\(_{50}\) dose of 10\(^8\) CFU of \(Fr.\) LVS, indicating a protective role for myeloid IKK\(\beta\) during overwhelming sepsis. IKK\(\beta\) deficiency also resulted in a dose-dependent survival response, an effect which has been observed, to varying degrees, for other components involved in NF-\(\kappa\)B signaling. For example, loss of either TLR2 (Toll-like receptor 2) [62] or MyD88 [55] results in reduced survival as a result of tularemic infection. Yet, loss of TLR2 affects survival at moderate infection doses (\(\sim 4 \times 10^4\) CFU) [62], while MyD88 deficient mice are extremely sensitive and succumb to \(Fr.\) LVS at infection doses orders of magnitude lower (\(5 \times 10^4\) CFU) [55]. The increased sensitivity of MyD88 loss is due to its requirement in multiple signaling pathways. Studies show that MyD88 loss affects the NF-\(\kappa\)B, c-Jun and p38 MAPK pathways with delayed kinetics [63], abrogated IL-1R- and IL-18R-mediated cytokine production [64], as well as impaired IFN-\(\gamma\) and TNF-\(\alpha\) production in response to several pathogens [63,64]. In addition, it is important to note that the dose-specific sensitivity to \(Fr.\) LVS in the TLR2 and MyD88 studies were performed in full knockout models, while the IKK\(\beta\) model used here was conditionally restricted to the myeloid compartment. Thus, this is the first study to show a myeloid-specific requirement for host survival in response to \textit{Francisella tularensis}.

\textit{Fr.} LVS Disseminates to Lung, Liver and Spleen

Dissemination of bacteria led to bacterial colonization in lung, spleen and liver tissues. Only mild histopathological findings in lung tissue were found. One unexpected finding was the development of organizing lymphocytic aggregates within the lungs IKK\(\beta\) cKO mice after infection. These aggregates, also known as iBALT (inducible bronchus-associated lymphoid tissue), are not present in normal lung in humans or mice, but can be induced after exposure to antigen, inflammation or infection (reviewed in [65,66]). In mice lacking spleen, lymph nodes and Peyer’s patches, iBALT acts as a site of B and T cell proliferation in response to influenza infection and is considered protective in nature [54]. iBALT develops under pathologic states, and it is suggested that this may involve the activation of PRR (pattern recognition

Figure 8. Loss of IKK\(\beta\) induces temporal M1 activation in response to \(Fr.\) LVS. Hepatic NPCs were isolated from IKK\(\beta^{\text{f/f}},\) IKK\(\alpha\) and IKK\(\beta\) cKO mice infected with 10\(^6\) CFU of \(Fr.\) LVS i.d. and analyzed by flow cytometry for M1 activation. (A) Pro-inflammatory macrophages were identified by their F4/80 and macroasin (CD68) expression and then scored for M1 activation by (B) IL-12 expression. Statistical analysis was performed by one-way ANOVA followed by Tukey’s ad hoc post-test. (n for IKK\(\alpha^{\text{cKO}},\) IKK\(\beta^{\text{cKO}}\) on day 0:5/5/5; day 2:4/4/4; day 6:4/4/4; day 8:4/4/3). Results are representative of at least two independent experiments. Bars represent the mean ± SD. **\(P<0.01\). doi:10.1371/journal.pone.0054124.g008
receptors) such as the TLRs [65]. Although further examination into the development of iBALT structures in IKKβ cKO mouse lung was beyond the scope of this current investigation, it is of note that these structures were previously identified in a number of Ft. LVS vaccine studies. Organized lymphoid structures are induced upon aerosol or intranasal administration of vaccines using whole [67], inactivated Ft. LVS [68] or Ft. LVS LPS plus adjuvant [69]. However, unlike these studies, where mice were immunized with several repeated doses of bacteria, our model consisted of a single intradermal inoculation. It is possible that the increased bacterial burden in IKKβ cKO mice, induces these structures as a result of ongoing and repeated exposure to Ft. antigen. Furthermore, iBALT structures persist in these mice well after bacterial clearance and are still evident at 90 days post infection (S.S., unpublished data).

IKKβ cKO mice responded to infection with an increased frequency in granuloma response in both spleen and liver. IKKα cKO mice developed some abnormally large granulomas within liver; however, the spleen granuloma reaction was qualitatively and quantitatively similar to IKKβ cKO mice. Analysis of Ft. antigen in the liver showed the bacteria were mainly localized within granulomas, although a limited number of infected cells could be detected within the parenchyma.

In the liver, iNOS (inducible nitric oxide synthase) is produced by macrophages and hepatocytes and can be induced by microbial lipoproteins through TLR activation, IFN-γ or TNF-α. iNOS mediates the metabolic conversion of L-arginine to NO (nitric oxide) and citrulline. NO is not only a powerful antimicrobial effector molecule capable of direct killing, it is also involved in several other aspects of host defense including cytokine production and apoptosis [70]. iNOS expression within granulomas was previously reported to be low and infrequent during intradermal infection [24]. However, we found 40–54% of hepatic granulomas were positive for iNOS induction by day 2, with only marginal changes in expression throughout the course of infection. Thus, it is unlikely that the observed defects in granuloma maintenance result from aberrant iNOS induction.

Apoptosis is considered an important immune defense mechanism. Rapid elimination of infected cells helps prevent the spread of infection to other cells and also promotes dendritic cell uptake of apoptotic bodies, allowing access to antigen which may trigger both innate and adaptive immune responses. However, many

**Figure 9.** Switch to M2a polarization in IKKβ cKO mice at mid-infection stage. M2a activation occurs in IKKβ cKO mice after sublethal i.d. infection with $10^6$ Ft. LVS as evidenced by expression of (A) CD206 (B) IL-10 (C) FIZZ1/Relmβ and (D) Arg-1 in a flow cytometry time course experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey’s ad hoc post-test. (n for IKKα f/f, IKKα cKO, IKKβ cKO on day 0/5/5; day 2/4/4; day 6/4/4; day 8/4/3). Results are representative of at least two independent experiments. Bars represent the mean ± SD. **P<0.01. doi:10.1371/journal.pone.0054124.g009
Pathogens have evolved mechanisms to escape destruction by subverting or redirecting these processes (reviewed in [71]). We found the early apoptosis marker, cleaved caspase-3 (CC-3), spatially localized within the granulomas in all three strains of mice. We found no overt differences between strains in the percentage of CC-3-positive granulomas at early time points.

Figure 10. Protracted hepatic activation of CD8 T cells in IKKβ cKO F. LVS infected mice. Hepatic (A) Natural killer (NK1.1+CD3−), (B) CD4+CD3+ and (C) CD8+CD3+ T-lymphocytes and their (D–F) IFN-γ expression, respectively, were analyzed by flow cytometry in F. LVS infected mice. Statistical analysis was performed by one-way ANOVA followed by Tukey’s ad hoc post-test. (n for IKKδ/−, IKKα cKO, IKKβ cKO on day 0:5/5/5; day 2:4/4/4; day 6:4/4/4; day 8:4/4/3). All results are representative of at least two independent experiments. Bars represent the mean ± SD. *P < 0.05; **P < 0.01.
Furthermore, only a few cells per granuloma stained positive for this marker, but this was consistent with other models of intradermal F. LVS infection [31]. Lawrence et al suggests that increased bactericidal control in a GBS model of pneumonia observed in \textit{tdx} \textit{AA/AA} macrophages is due, in part, to increased activation of anti-apoptotic genes combined with sustained activation of inflammatory genes such as iNOS [10]. In the \textit{Ft. LVS} model, these effects on apoptosis and iNOS production were not evident, suggesting that the molecular mechanisms between these two infection models are different. Furthermore, GBS-infected \textit{tdx} \textit{AA/AA} mice, despite increased bacterial clearance at early time points, eventually succumb to infection, whereas survival in IKK\textit{a} cKO mice infected at the LD\textsubscript{50} dose of 10\textsuperscript{6} CFU of \textit{Ft. LVS} are similar to wild type mice.

**IKK\textit{b} Deficiency in Myeloid Cells Causes Time Dependent, Differential Effects on M1 vs M2 Macrophage Polarization in Response to Sublethal \textit{Ft. LVS} Infection**

Despite differences in survival, the observation that myeloid IKK\textit{b} deficiency induces M1/classical activation in the livers of \textit{Ft. LVS} infected mice is in accord with the GBS infection model [11]. Because our model system allows us to monitor macrophage polarization over days, not hours (as is the case in GBS model), we also discovered that IKK\textit{b} cKO mice undergo a second wave of polarization toward M2/alternatively activated macrophages at the mid-infection stage. Although we cannot rule out that this second wave of polarization stems from secondary or bystander effects induced by the local environment, it is of note that the anti-inflammatory IKK\textit{b}-dependent blockade of M1 classical macrophage activation is transient in nature and thus an important consideration for design of therapeutic targeting.

**IKK\textit{b} Loss in Myeloid Cells Results in Collateral Effects on IFN-\gamma Producing Cells**

The compounding effects of increased bacterial titers and M1/ M2 activation defects in IKK\textit{b} cKO mice also correlated with increases in several types of IFN-\gamma expressing cells. Furthermore, prolonged elevations in IFN-\gamma expressing CD8\textsuperscript{+} T cells were protracted throughout the course of infection. Elevations of these cells persisted, despite the mid-infection shift in M2 macrophage polarization. It has long been considered that early tularemic infection is controlled by T cell-independent events, as T cell depleted mice (depleted of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells) survive initial infection, but fail to clear bacteria and succumb to infection several weeks after inoculation [72,73,74]. Interestingly, mice selectively depleted for only CD4\textsuperscript{+} T cells or CD8\textsuperscript{+} T cells are able to resolve and survive infection [75]. This implies that these T cell subsets have roles in clearance and resolution of infection that largely overlap. Although the exact mechanism(s) by which myeloid IKK\textit{b} deficiency results in a collateral elevation of reactive CD8\textsuperscript{+} T cells remains to be determined, our data suggest that when myeloid NF-kB is impaired due to loss of IKK\textit{b}, CD8\textsuperscript{+} T cells overly respond as an immunologic compensatory mechanism. Therefore, it seems that CD8\textsuperscript{+} T cells are poised to react during the T-cell independent phase of \textit{Ft.} infection.

In summary, we show that myeloid IKK\textit{b} has a protective role in controlling bacterial growth that leads to overwhelming sepsis during tularemic infection. Furthermore, macrophage polarization vs. bacterial control and survival appear to be functionally distinct processes regulated by myeloid IKK\textit{b}. In addition, we also found, in our model system, that IKK\textit{b}-dependent macrophage polarization is a short-term phenomenon. Contrary to this, we found no evidence of anti-inflammatory properties for myeloid IKK\textit{b} in vivo.

**Supporting Information**

Figure S1 LD\textsubscript{50} and sublethal \textit{Ft. LVS} dose determinations. (A) IKK\textit{f/f} control mice (n = 5 mice/group) were injected with \textit{Ft. LVS} i.d. at the indicated doses and analyzed for survival by Kaplan-Meier method in order to determine the median lethal dose. The inoculation dose was confirmed by retroplate assay and the LD\textsubscript{50} was estimated at 10\textsuperscript{6} CFU. In panels (B) and (C), IKK\textit{f/f}, IKK\textit{a} cKO and IKK\textit{b} cKO mice were injected with \textit{Ft. LVS} i.d at two different sublethal doses: (B) 10\textsuperscript{4} CFU and (C) 10\textsuperscript{6} CFU, and analyzed for long-term survival (n = 5–6 mice/group). (TIF)

Figure S2 Minimal lung involvement after intradermal \textit{Ft. LVS} challenge. Lung sections, taken from mice i.d. challenged with a sublethal dose of 10\textsuperscript{6} CFU \textit{Ft. LVS}, were analyzed by immunohistochemistry for \textit{Ft. LVS} antigen. (A) and (D) IKK\textit{f/f}, (B) and (E) IKK\textit{a} cKO, and (C) and (F) IKK\textit{b} cKO are representative lung sections showing low \textit{Ft. LVS} colonization at days 2 and 9 post infection, respectively. Magnification = 100x, scale bar = 100 \textmu m. (TIF)

Figure S3 \textit{Ft. LVS} dissemination in spleen. Spleen sections from mice i.d. challenged with a sublethal dose of 10\textsuperscript{6} CFU \textit{Ft. LVS}, were analyzed by immunohistochemistry for \textit{Ft. LVS} antigen. (A) and (D) IKK\textit{f/f}, (B) and (E) IKK\textit{a} cKO, and (C) and (F) IKK\textit{b} cKO are representative sections of spleens at days 2 and 9 post infection, respectively. Magnification = 100x, scale bar = 100 \textmu m. (TIF)

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**Author Contributions**

Financially supported this research with his awarded grants: KBM. Conceived and designed the experiments: SS. Performed the experiments: SS. Analyzed the data: SS. Contributed reagents/materials/analysis tools: SS KBM. Wrote the paper: SS KBM.

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