Follistatin-like protein 1 deficiency confers protection from *Klebsiella pneumoniae* pulmonary infection

by

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Abstract

Klebsiella pneumoniae is the third most common cause of nosocomial infections and leads to tremendous burden on health care spending and infection control, due to multiple drug resistance to the majority of present-day antibiotics. Elucidating the role of diverse innate immunity control mechanisms and signaling pathways *in Klebsiella pneumoniae* host response is essential in public health importance for developing new therapeutic antibiotic-alternatives. There is currently a gap in the research literature about the role of Follistatin-like protein 1 in infectious diseases. In this study, we establish that innate mechanisms can play a protective role in a *Klebsiella pneumoniae* pulmonary infection murine model via decreased secreted glycoprotein Follistatin-Like 1 Protein (FSTL1). Using a FSTL1 hypomorphic *in vivo* mouse model, we discovered that deficiency in FSTL1 production leads to significantly lower *Klebsiella pneumoniae* infectious burden ($p<0.005$) in a lung infection model. We additionally assessed gene expression by real-time qPCR that this lower burden was associated with differences in pro-inflammatory cytokine signaling, as expression of *il7a, il1b, and csf3* were all significantly upregulated in FSTL1 hypomorphic mice compared to C57B6 wildtype controls. We further identified that FSTL1 hypomorphic mice express significantly higher γδ T cell receptor components, *trdc* ($p<0.001$) and *trv4* ($p<0.005$), as well as increased populations of γδ T cells with significantly higher in IL-17A cytokine production ($p<0.05$) via flow cytometry analysis. These phenotypes were not able to be phenocopied in

FSTL1 conditional knockout mice or the FSTL1 neutralizing antibody experimental model, suggesting that FSTL-1 hypomorph mice, uniquely, have increased γδ T-cells populations that likely are essential for control of infection. Our study provides evidence that deficient FSTL1 production in genetic and developmental mouse model can protect against *Klebsiella pneumoniae* lung infection. If this discovery is translational to the human condition, it could signify further research into FSTL1 protein as a host-oriented broad-spectrum drug target is warranted.

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Preface

Throughout my research career, I have had several prominent mentors that have come into my life and continue to check up with me to see how my researcher career is unfolding. I consider each of them a gift for the invaluable time they have placed into developing my research skills, critical thinking skills, and confidence in my ability to fulfill my career goals.

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1.0 Introduction

1.1 Antimicrobial Resistance Burden

The overuse of antibiotics has led to a global epidemic of antimicrobial resistance. Untreatable infections account for 25,000 deaths per year across the EU [1]. The economic impact of antimicrobial resistance is estimated to reach 10 million deaths per year and cost the world up to $100 trillion [1]. Antimicrobial resistance can be driven by over prescription of antibiotics; healthcare providers have been attributed to have a rate of 836 antibiotic prescriptions per 1000 persons in the US [2]. Interventions for inappropriate over prescription of antibiotics in the health care setting can help solve the problem, but it may not be enough. Indeed, physicians reduced antibiotic prescription for uncomplicated bronchitis from 74 to 48% by implementing office and household education materials for patients and education intervention [3]. There are additional reasons for the development of high antimicrobial resistance burden. Concentrated feeding animal operations (CFAOs) allow for the rapid spread of genetic resistance because animals are housed in concentrated environments and antimicrobials are supplemented into feed [4].

Klebsiella Pneumoniae is the most common transmissible Carbapenemase-producing Enterobacteriaceae (CPE) worldwide [5]. These strains of bacteria produce the beta-lactamase hydrolyzing enzymes that have the ability to resist carbapenems. Additionally, Klebsiella

pneumoniae can be resistant to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole typically used in combating these gram-negative infections Carbapenemase-producing strains specifically are associated with a 40-50% mortality rate [5]. The genes allowing for the production of B-lactamases are located on the bla_{KPC} plasmid, which can be horizontally transferred between strains of *K. pneumonia* and other gram-negative bacteria in and during the formation of biofilms [6].

Once antimicrobial resistant infections are identified, they must be contained to decrease the spread of infection to vulnerable populations, especially in the health care setting. The Center for Disease Control has issued guidelines specifically for Carbapenem-Resistant and Carbapenemase-Producing Enterobacteriaceae that can be applied towards containing *Klebsiella pneumoniae* infections [7]. Because the pathogen is spread via person-to-person contact, the CDC recommends establishing isolation precautions for patients and medical staff such as disposable gowns and gloves. It is recommended that the clinical microbiology labs follow the Clinical and Laboratory Standards for susceptibility testing and establish a protocol for carbapenemase detection. It is also recommended that hospitals review clinical culture results for the past 5-12 months to find any unrecognized CRE [7]. Additionally, the Infectious Disease Society of America has recommended the Food and Drug Administration to consider reducing regulations on effective drug development. The Society argues that it would be better to have a lower effective drug against these infections than none at all [8], where we currently stand due to widespread antimicrobial resistance.

1.2 *Klebsiella Pneumoniae* in Human Host

Klebsiella pneumoniae (K. pneumoniae) is a Gram-negative, rod-shaped bacterium that is lactose-fermenting, non-motile, aerobic and mucoid. It has a prominent capsule surrounding its bacterial cell wall that is about 160 nm thick and helps the bacterium survive harsh host environments [9]. The serotype of the pathogen is associated with the repeating polysaccharide subunits that make up the capsule. These sugar subunits are repeating linear units and can be made up of di-mannose/rhamnose residues, D-glucuronic acid, D-galactose, D-mannose, and L-rhamnose, among others [9]. There are over 77 varieties of K antigen and 9 varieties of O antigen of the lipopolysaccharide that determine serotype. The K1 serotype is most commonly associated with pyogenic infections and K2 serotype is associated with UTIs (13% of clinical isolates) but there is a broad range of K2 serotypes [10].

K. pneumoniae possesses several pathogenicity factors that promote an ability to evade host immunity. The capsule is a central component for biofilm formation and complement resistance which increases the pathogenicity of the bacterium [11]. The thick lipopolysaccharide capsule of the pathogen allows for evasion of phagocytosis and the complement system. Specifically, a hypermucoviscocity phenotype exists if the bacteria serotype is able to produce a mucoviscous exopolysaccharide web [12]. The hypermucoviscous strains grow in sticky colonies and have a positive string test if a bacteriologic loop can stretch a viscous string from the colony for greater than 5 mm. K1 and K2 serotypes are more hypermucoviscous than non-K1/K2 strains and can be more clinically invasive [12].

The host complement system is comprised of more than thirty proteins produced in the liver and activated macrophages. A cascade of reactions results in the conversion and cleavage of complement proteins to promote opsonization and the formation of the membrane-attack

complex, or MAC. The three complement system pathways include the classical, alternative, and lectin pathways. One mechanism for which *K. pneumoniae* avoids complement system killing is by not having mannobiose or rhamnobiose glycan subunits in the capsule, targets of the mannose-binding lectin complement pathway [9]. The pathogen can modify the lipid A side chain of lipopolysaccharide that anchors to the cell membrane during infection and avoid host system toll-like receptor 4 stimulation by LPS [9].

K. pneumoniae strains with long O-antigen side chains can also avoid the complement system. These strains are considered to have smooth LPS rather than rough LPS. The long O-antigen side chain disrupts binding of C1q binding and makes C3b complement component for opsonization bind farther away from the surface of the bacteria. This mechanism decreases the chances of membrane attack formation [9]. The capsule can also induce dendritic cell maturation with increased surface markers CD83, CD86, and TLR4 but decreased CD14 creating a defective immunological response [10].

Siderophores are used by *K. pneumoniae* as another survival mechanism. Siderophores are cation chelators that allow for the uptake of iron by the bacteria [14]. Iron is an essential resource needed for several bacterial metabolic processes and bacterial growth. All K1 and K2 *Klebsiella pneumoniae* strains have the Enterobactin iron-uptake siderophores [10]. The mammalian host can overcome the this siderophore survival mechanism via upregulation of lipocalin2. Lipocalin2 outcompetes the siderophores by binding to and inactivating them. *K. Pneumoniae* infection induces the cytokine IL-22 response which upregulates lipocalin2 and enhances the host defense [15]. Recently, research has shown that siderophores not only chelate iron but can also contribute to the secretion of pro-inflammatory cytokines and dissemination of bacteria into the spleen, therefore furthering infection severity [14].

Klebsiella pneumoniae is an opportunistic pathogen that is carried in the community at a 5 to 38 percent rate in stool and 1 to 6 percent rate in the nasopharynx [12]. *K. pneumoniae* is normally carried asymptomatically in the stool, nose, throat, and skin. The pathogen is more prevalent in the hospital setting than the community and is responsible for about 3 to 8 percent of all nosocomial bacterial infections [12]. *Klebsiella pneumoniae* is spread by person-to-person transmission, but not through the air, and manifests itself as respiratory pneumonia infection, urinary tract infection, wounds, bacteremia, sepsis and liver abscesses [10].

K. pneumoniae based infections were originally linked to immunocompromised patients who presented with aspiration pneumonia. The highest risk population for these infections is now known to be a wide range of immunocompromised persons that includes individuals with diabetes mellitus, chronic obstructive pulmonary disease, renal failure, organ transplantations, and cancer [10]. Specifically, individuals with invasive plastics such as urinary tract catheters and intravenous catheters are at high risk for infection because the pathogen can create a biofilm on these devices allowing access to otherwise sterile body sites [9]. Persons with these risk factors can frequently be found in the hospital and long-term acute care settings. These populations of patients have higher rates of antibiotic use that can facilitate colonization of the bacteria. *K. Pneumoniae* infections can occur anytime throughout the lifespan, but the elderly and neonates are notable susceptible populations. These populations need varying amount of water for their cells and have limited access to said water in order to maintain an osmolality balance for healthy immune systems to fight off infections [11].

1.3 Antimicrobial Resistance via Biofilms

As mentioned, patients with invasive plastics such as catheters are at higher risk for infection by *Klebsiella pneumoniae*. Biofilms are slimy layers of bacteria that adhere to surfaces, abiotic or biotic. *K. pneumoniae* uses type iii fimbriae that express the MarkA and MarkD adhesion proteins. The MarkA adhesion protein aids in the formation of biofilms on abiotic surfaces and the MarkD adhesion protein aids in the formation of biofilms in extracellular human matrix[12]. The type iii fimbriae, bacterial capsule, and lipopolysaccharide factors together allow for the formation of the biofilm [12]. The formation of biofilms can involve single-organism *Klebsiella pneumoniae* colonies or be mixed with other bacteria such as *Proteus mirabilis, Morganella morganii, Enterobacter cloacae, Pseudomonas aeruginosa and Pseudomonas protegens* [12]. The formation of biofilms allows the bacteria to increase growth at a much steeper rate and exchange virulence factors to increase pathogenicity. Virulence factors such as antibiotic resistance, especially B-lactam carbapenemase, can be exchanged via plasmids in the complex biofilm environments, which further contributes to increased morbidity and mortality. A biofilm is more resistant to antibiotics than bacteria alone because the dense matrix of proteins, polysaccharides, and DNA prevents diffusion of the antibiotics resulting in less exposure to the pathogen [10].

1.4 Host-oriented Broad-Spectrum Drug Targets (HOBS)

Due to the nature of resistance to antibiotics, antibiotic alternative therapies need to be researched and created for the general population and immunocompromised subpopulations. Secondary metabolites from plants have potential for new drug development against bacterial infections because of their bioactive nature [16]. However, another strategy with higher potential is host-oriented broad-spectrum drug targets [17].

Modifications to host response factors could include designing drugs or therapies to augment innate and adaptive immunity responses to infection. One advantage of host response modifications is the intrinsic reduced potential for bacterial species to acquire resistance. The host modifications such as protein production can have redundancy in pathways to reach the same effect. So, if a bacteria species is able to acquire resistance against the host protein itself, it still has to work against the down streaming signal pathways as well. One potential host-oriented broad-spectrum drug target is Follistatin-like protein 1 (FSTL1).

1.5 Follistatin-like Protein 1 (FSTL1) Protein Biology

Follistatin-like protein 1 is an 11-exon protein (Sundaram et al) which consists of 308 amino acids [18]. Follistatin-like protein 1 is a member of the secreted protein acidic rich in cysteins (SPARC) family of proteins because possesses a follistatin-like domain and an extracellular calcium binding E-F hand motif [19]. However, unlike the other eight members of the SPARC family, the extracellular calcium binding domain has been deemed nonfunctional. The

SPARC family consists of eight proteins that are known to be secreted into the extracellular space [19].

Follistatin-like protein 1 is widely expressed throughout the human transcriptome with RNA-seq analysis showing higher expression in the adrenal, prostate, gall bladder, urinary bladder, placenta, and fat tissues. FSTL1 is expressed in the lung with one study showing a medium peak of 55.776 ± 16.707 Reads Per Kilobase of transcript among five normal human tissues [20]. Additionally, FSTL1 is important in lung development. The *Fstl1*$^{-/-}$ global mouse knockout is perinatal lethal, likely due to multiple developmental anomalies; notably *Fstl1*$^{-/-}$ are cyanotic at birth due to collapsed lungs and lack of air in the distal airways [21]. FSTL1 has been shown to be a BMP4 antagonist that results in downregulated surfactant expression and tracheal ring development [21].

Overall, relatively little is known about the molecule FSTL1 and there is evidence to support multiple, and often contradictory, functions in inflammation and mammalian biology. As of April 2019, a PubMed search for FSTL1 encompasses only 191 total results. Additionally, there is contention in the field about whether FSTL1 acts dominantly as a pro-inflammatory or anti-inflammatory signaling molecule [22]. FSTL1 treatment has been shown to decrease the pro-inflammatory cytokines IL-6 and c-fos in collagen-induced arthritis [23]. Additional anti-inflammatory evidence exists in heart allograft tolerance, where FSTL1 overexpression inhibited the pro-inflammatory cytokines IL-6, IL-17A, and IFNγ [24]. However, FSTL1 has been shown to be pro-inflammatory in obesity via 3T3-L1 adipocytes and RAW264 macrophage *in vitro* experiments. FSTL1 increased pro-inflammatory cytokines IL-6, TNFα, and MCP-1 in these cells [25]. There is currently a gap in the literature about the involvement of FSTL1 in infectious

diseases. One study found that patients with bacterial sepsis had a three-fold increase in FSTL1 serum concentration. Additionally, macrophages were able to take up FSTL1 in mice [22].

FSTL1 has the potential to provoke several immune cell types in a pro-inflammatory manner. Macrophages, neutrophils, basophils, mast cells, and eosinophils are responsible for producing cytokines in response to a Pathogen-associated molecular patterns of the foreign invader and developing further adaptive responses, like Th17 cells in response to bacterial and fungal infections. These cytokines provoke specific T helper cells to come to the scene of infection through dendritic cells and other antigen-presenting cells. Gamma delta T cells and Natrual killer T cells fall in the “in between” or “overlapping category between innate and adaptive immunity. In contrast to ab T cells, gamma delta (γδ) T cells have a TCR that is made up of one γ (gamma) chain and one δ (delta) chain. Gamma delta T cells they do not seem to require antigen processing and major-histocompatibility-complex (MHC) presentation of peptide epitopes. These cells are also capable of phagocytosis [26].

2.0 Statement of Project and Hypothesis

My project focused on answering the question: Does Follistain-like protein 1 (FSTL1) play any type of immunomodulatory role during *Klebsiella pneumoniae* pulmonary infection? In-vivo mouse models were used for FSTL1 protein modeling due to the high degree of homology between the mouse and human FSTL1 protein. The overall goal of the project is to identify whether there is a phenotype of differential *K. pneumoniae* bacterial clearance from the lung due to FSTL1 protein interaction in the lung. Several in-vivo mouse models to address this question were implemented. The FSTL1 hypomorphic mouse models the effect of reduced FSTL1 transcription from the germline stage (including development). FSTL1 conditional knockout models and neutralizing antibody address the question about how decreasing FSTL1 genes and protein, respectively during *Klebsiella pneumoniae* infection can affect lung bacterial clearance. Recombinant FSTL1 protein modeling was used to address the question of how exogenous/supranormal FSTL1 protein only during *Klebsiella pneumoniae* infection can affect lung bacterial clearance.

I hypothesized that in a *Klebsiella pneumoniae* pulmonary infection will have decreased lung burden by FSTL1 signaling. FSTL1 will signal for the recruitment of innate immune cells and produce more inflammation leading to clearance of *Klebsiella pneumoniae.* Further mouse modeling into cell type and mechanism for bacterial clearance was completed after a phenotype was established.

The current gaps in the literature about the role of FSTL1 infectious diseases required initial studies to refine the model, specifically: CFU inoculum, FSTL1 conditional knockout

models, recombinant FSTL1 dosing, and anti-FSTL1 antibody dosing concentrations and served as the main focus of my work.

3.0 Public Health Relevance

Finding alternative therapies to multi-drug resistant bacterial infections is essential for overall population health. The evolution of the history of bacterial resistance to present-day antibiotics suggests that current and yet-to-be developed antibiotic therapies have the potential to become obsolete in short periods of time. In order to maximize efficacy of future treatments in the prevention of multidrug resistant bacterial infections, including efficacy for *Klebsiella pneumoniae*, scientists must look into alternative therapies that focus on maximizing human host immunity. One such treatment could be upregulation or downregulation of the human gene Follistin-Like Protein 1. If Follistatin-like Protein 1 is able to affect *Klebsiella pneumoniae* bacterial clearance during pulmonary lung infection, then it can serve as a Host-oriented Broad-Spectrum Drug Target (HOBS) for subpopulations that can utilize the therapy. Host-oriented broad-spectrum drug targets may be beneficial treatments for multi-drug resistant bacteria if the bacteria take a longer time to develop resistance, if they are able to at all. Maximizing prevention and treatment mechanisms for immunocompromised persons most susceptible to drug-resistant *Klebsiella pneumoniae* infection can increase the overall population health of hospital care facilities and decrease the global burden of antimicrobial resistant infections and their economic burden on healthcare.

4.0 Research Methods

4.1 Ethical Statement

This work was approved by the Institutional Review Board and the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh. All animals were euthanized humanely and we are appreciative of their contributions to answering our scientific questions.

4.2 Animal Models

All *in vivo* experimentation for this study was performed in the Rangos Research Building Animal Facility using various mouse models on the C57/B6 background. Procedures were performed under the IACUC protocol 17081376. All animals were sex-matched for experimentation and housed randomly per cage, expect for in the case of the FSTL1 conditional knockout model where sexes were evenly distributed between the knockout model group and C57/B6 wild-type control groups. All animals used were between the ages of 9-14 weeks of age. C57/B6 control, B6.129P2-Tcrdtm1Mom/J, and B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred to create each respective mouse model. FSTL1 hypomorphic mice were a gift from Dr. Raphael Hirsch and previously characterized by Dr. Brian Campfield as expressing around 60% FSTL1 protein from birth [27]. The FSTL1 conditional knockout model described is the result of a breeding cross between B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J mice and FSTL1 floxed mice where the

FSTL1 gene is floxed out by tamoxifen-inducible Cre-recombinase expressed at the *Rosa26* locus. The animal described as "Both Knockout" is the result of the breeding of FSTL1 Hypomorphic mouse with B6.129P2-Tcrdtm1Mom/J.

4.3 *Klebsiella pneumoniae* Inoculation

Klebsiella pneumoniae strain 396, a K1 serotype, was used for all experiments with a desired inoculation of $1x10^4$CFU per mouse. Klebsiella pneumoniae was grown in Trypticase soy broth (TSB) and incubated overnight at 37°C and 250RPM. *Klebsiella pnemoniae* was then sub-cultured in 2ml TSB the next day in order to reach bacterial log phase with the goal concentration of 1E9 CFU/ml. Sub-cultured Klebsiella pneumoniae was spun down at 5000G for 5 min and supernatant was aspirated off. Sub-cultured Klebsiella pneumoniae was resuspended in 1ml of PBS. Sub-cultured *Klebsiella pneumniae* was then diluted 1:5000 in PBS and vortexed. 50ul of $2x10^5$ inoculum was administered to mice intratracheally under 1:1 isoflorane via tongue-pull technique. Inoculum concentration was verified by dilution series and LB plate Colony Forming Units (CFU) calculation.

4.4 Tissue Extraction Colony Forming Units

At experimental endpoints, mice were sacrificed using isoflurane and secondary terminal bleed. Right lung lobes were extracted and separated into 1ml PBS. Liver and spleen were also

extracted and separated into 1ml PBS, depending on experiment. Organs were homogenized, serially diluted, and 10ul of dilutions streaked onto LB agar plates. Bacterial colonies were counted after 6 hours incubation at 37°C.

4.5 Tissue Processing for RNA

Left lung lobes were extracted and homogenized into 1ml Trizol (Life Technologies) and RNA was isolated per manufacturer's instructions. Nanodrop 2000 (Thermofisher Scientific) was used to quantify RNA and the ratio of absorbance at 260 nm and 280 nm was used to determine RNA purity. Samples less than 2.0 for this ratio were regarded as impure and not used for analysis. 40ng RNA was converted to cDNA using iScript (Biorad) and genetic expression assays were performed using Reverse Transcript Supermix (Biorad) or Taqman mastermix (Applied Biosystems) depending on primer probe. Primer probe assays were from ordered from Applied Biosciences or IDT.

4.6 Flow Cytometry

Lung cells were prepared for flow cytometric analysis by collecting middle right lobe of lung, followed by 4mg/mL Collagenase (Sigma cat# C0130) and 0.2mg/mL Dnase (Sigma cat# DN25) digestion at 37^{o}C with agitation for 1 hour and straining digest through a 70um filter. Strained cell pellets were collected by centrifugation at 500xg for 5 minutes and red blood cells were lysed using ACK Lysing Buffer (Gibco cat# A10492-01) according to manufacturer

recommendations. Cells were then resuspended in 1X Phosphate Buffered Saline (PBS) for transition into the flow cytometry cell staining protocol. After cells were stained with cell surface markers of interest, the cells were washed in 1XPBS, Fixed for 30min on ice using 1X Fix Buffer, washed with 1XPerm Wash Buffer, and stained with intracellular antibody cocktail in 1X perm wash buffer for 30-60min on ice. For data acquisition (phenotypic analysis), cells underwent flow cytometry on an LSR Fortessa (BD Biosciences) using FACSDiva software version 8.0.1 (BD Biosciences). Data was analyzed using FlowJo software version 10.1 (BD Biosciences).

4.7 Reagents

Rabbit IgG (R&D, Catalog No. AB105C) and Follistatin-like 1/FSTL1 Rat anti-Mouse, Monoclonal (R&D/Fisher Catalog No. MAB1738) were administered intratracheal and intrapleural for the neutralizing experiments. Neutralizing experiment doses were 200ng Recombinant Mouse Follistatin-like 1 Protein (R&D Catalog No. 1738-FN-050) and Bovine Albumin Protein (Thermofisher Scientific Catalog No. 15260037) were administered intra-trachaelly for the recombinant protein experiments. For flow cytometry analysis, the panel included antibodies FITC-IL-17A (eBio 11-7177-81), PE-γδTCR (eBio 12-5711-82), PerCP-Cy5.5-CD8a (BD 561109), APC-Cy7-CD3 (BioLegend 100222), BV421-IFNy (BD 563376), and BV786-CD4 (BD 563727).

4.8 Statistical Analysis

Statistical analysis was performed using Prism 7 for Mac OS X, Version 7.0a graphing and statistical analysis software provided by the University of Pittsburgh. Experimental graphs were created in PRISM and figures constructed using Adobe Photoshop CC 2019.

5.0 Results

5.1 *K. pneumoniae* Infection in *Fstl1* Hypomorphic Mice

The first question addressed for this project was: Is there a difference in *K. pneumoniae* bacterial clearance for the FSTL1 Hypomorphic Mouse Model. The Wild-type C57B6 and FSTL1 Hypomorphic mice were weighed at the start of the infection in order to address weight as a possible confounding factor. Colony Forming Units can be affected by the weight of the animal, where having a higher weight may serve as a protection factor against bacterial burden. As shown in Figure **1A**, there were no significant differences between baseline weights among the two experimental groups. The Klebsiella pneumoniae lung burden between the two groups was statistically significant (**1B**), where $p<0.005$ by student's T test. FSTL1 hypomorphic mice have lower lung bacteria burden and are therefore protected against *K. pneumoniae* pulmonary infection at 48 hours of infection. Spleen bacterial burden was addressed using cultural plates for the neat level, and all animals had uncountable burden.

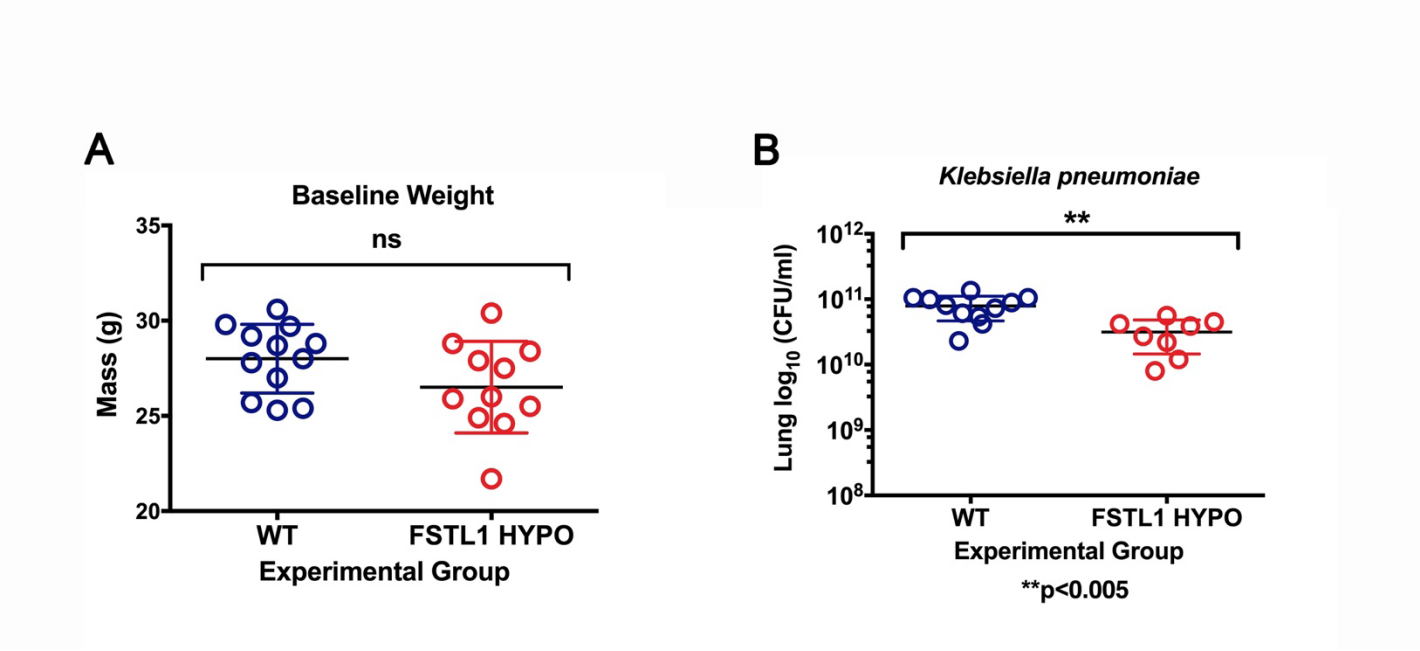

Figure 1 *K. pneumoniae* infection in FSTL1 Hypomorphic Mice

FSTL1 hypomorphic mice are protected against Klebsiella pneumoniae pulmonary infection at 48 hours of infection**. (A)** C57B6 and FSTL1 hypomorphic mice were of nonsignificant weight differences (p=0.1057) at the start of infection, as analyzed by unpaired Student's T-test. **(B)** Log10 CFU/ml was significantly reduced for FSTL1 hypomorphic mice (p=0.0015) by unpaired Student's T test compared to sex-matched, age-matched C57B6 Wildtype controls.

The next question addressed whether the difference in *K. penumoniae* bacterial burden due to pro-inflammatory cytokines? Because there was a no difference in *K. pneumoniae* lung burden after 24 hours **(2A),** we were able to address the transcript levels for cytokines without CFU being a confounding factor. Our FSTL1 hypomorphic mouse model was verified to be producing significantly lower levels of *fstl1* transcript (**2B**). The genes *csf3, il1b,* and il17a had significantly higher transcript levels in the FSTL1 hypomorphic mice compared to wild-type controls **(2D-F).** Additionally, there was a trend in higher transcript levels of *il6* in the FSTL1 hypomorphic mice compared to controls.

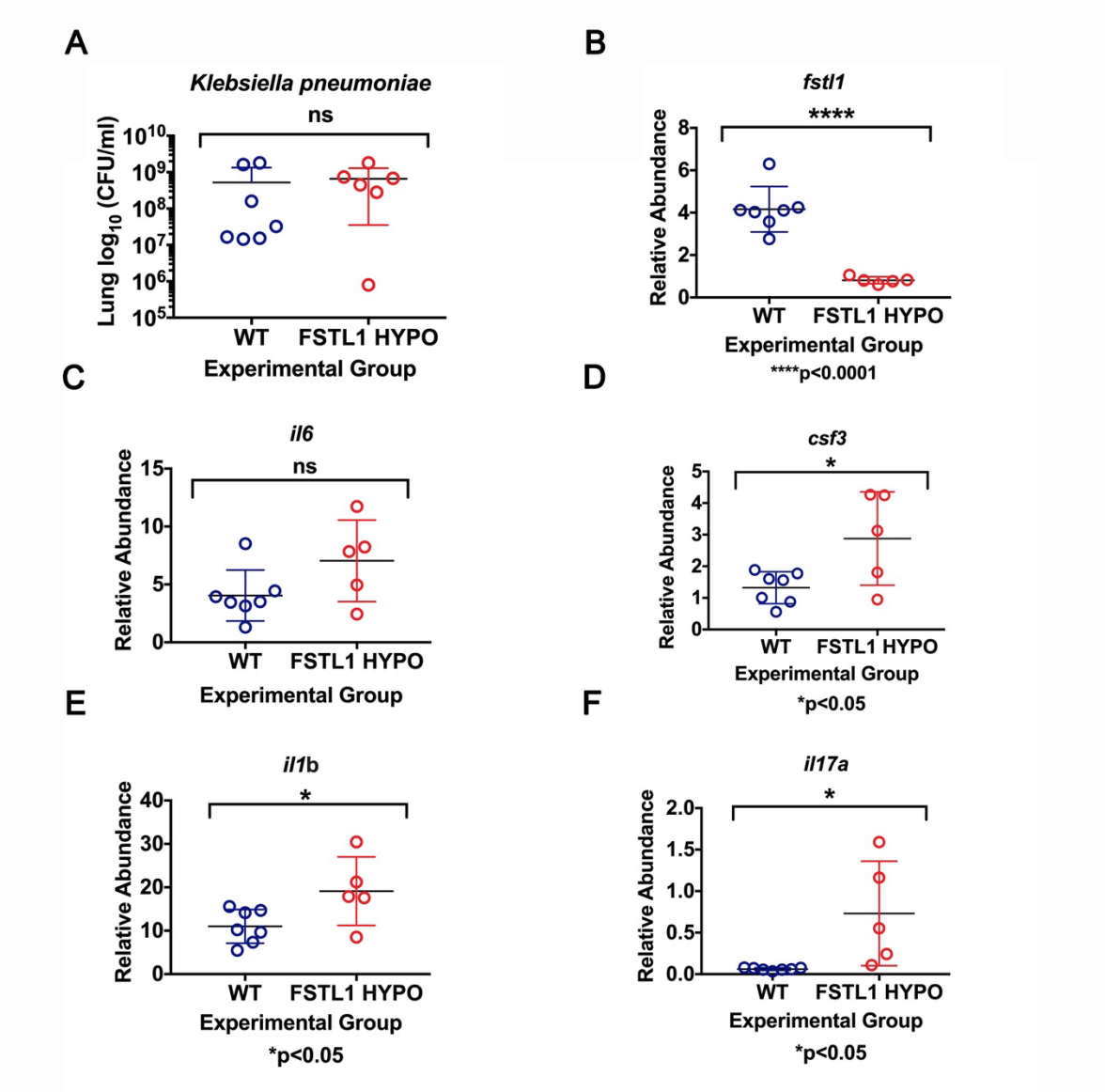

Figure 2. Cytokine Production in FSTL1 Hypomorphic Mice

FIGURE 2. FSTL1 hypomorphic mice have upregulated pro-inflammatory cytokine production at 24 hours of infection. **(A)** Colony Forming Units per ml between both FSTL1 hypomorphs and wildtype controls after 24 hour of initial 1x104 CFU inoculum via intratracheal instillation. Experimental groups were compared using unpaired Student's T test (p=0.7489). Because there were nonsignificant differences in CFU/ml among the groups, relative transcript abundance could be adequately measured. **(B)** Hypomorphic mice expressed significantly less (p<0.0001) fstl1 transcript compared to controls after 24 hours of KP infection. Relative abundance of fstl1 was compared to gapdh expression levels **(C)-(F)** Relative abundance of pro-inflammatory cytokine transcripts were assessed after 24 hours of KP infection and compared using unpaired Student's T test. Hypomorphic mice trended to express higher (p=0.0985) il6 transcript, significantly higher csf (p=0.0256) transcript, significantly higher (p=0.0388) il1β transcript, and significantly higher (p=0.0166) il17a transcript. Suggesting that a pro-inflammatory response is induced in a model where significantly less fstl1 transcript is expressed at start of infection. All transcripts were normalized to *hprt* or *gapdh* expression levels.

We next addressed the research question: What immune cells could be responsible for these pro-inflammatory cytokines? Using flow cytometry methods, we discovered that there was no significant difference between FSTL1 hypomorphic mice and WT controls for the percentage of cells CD8+ T cells or CD4+ T cells **(3A-C)**, but the γδ T-cell populations were significantly different after 48 hours of Klebsiella pneumoniae infection. We further verified that FSTL1 hypomorphic mice express significantly higher *trdc* **(3D)** and significantly higher *trdv4* **(3E)** transcript during Klebsiella pneumoniae pulmonary infection. With further flow cytometry analysis, we clarified that γδ T-cell populations, but not CD4+ or CD8+ T cell populations, are responsible for higher production of IL-17A cytokine in the FSTL1 hypomorphs compared to wildtype controls **(3F-H).**

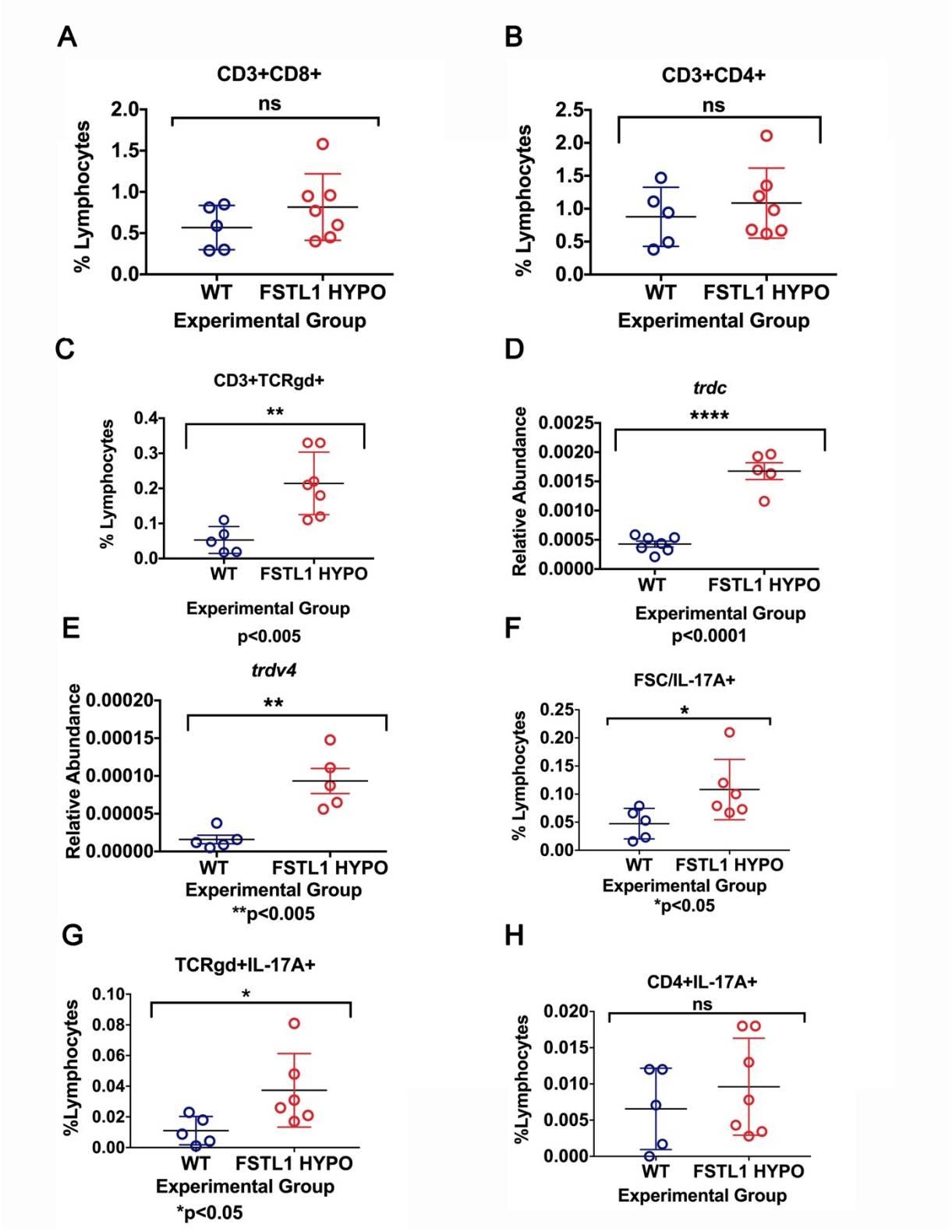

Figure 3. Evaluation of IL17A Producers in FSTL1 Hypomorphic Mice

FIGURE 3. FSTL1 hypomorphic mice highly express T cell receptor delta, constant region trdc and fetal Vδ gene segment trdv4. Gamma delta T cells in hypomorphic mice are IL17a producing in Klebsiella pneumoniae pulmonary infection.
(A)-(B) FSTL1 hypomorphic mice express significantly higher trdc ($p=0.0023$) and significantly higher trdv4 ($p<0.0001$) transcript during Klebsiella pneumoniae pulmonary infection, as analyzed via unpaired Student's T-test. Expression levels were compared to hprt form normalization **(C)** Overall, the percentage of lymphocytes that express il17a is significantly higher ($p=0.0479$) in the FSTL1 hypomorphic mice compared to controls, as analyzed by unpaired Student's T-test. **(D)** IL17a producing CD4+ T-cells are not significantly different ($p=0.4244$) between FSTL1 hypomorphic mice and controls, as analyzed by unpaired Student's T-test. **(E)** IL17a producing TCRgd T-cells are significantly higher ($p=0.0470$) in FSTL1 hypomorphic mice compared to controls, as analyzed by Student's T-test.

5.2 *K. pneumoniae* Infection in Delta Mouse Models

We then asked the question: If γδ T cells are the cell type responsible in the phenotype, what happens to bacterial burden if we knock them out? We hypothesized that knockoing out the γδ T cells and having a hypomorphic level of FSTL1 would decrease the lung bacterial burden. There were differences in lung bacterial burden among all three groups of mice (figure number) and no significant differences among spleen bacterial burden. Knocking out gamma delta T cell knockout mice have increased *Klebsiella pneumoniae* lung burden compared to FSTL1 hypomorphic, gamma delta T cell knockouts and FSTL1 hypomorphic mice after 48 hours of *Klebsiella pneumoniae* pulmonary infection.

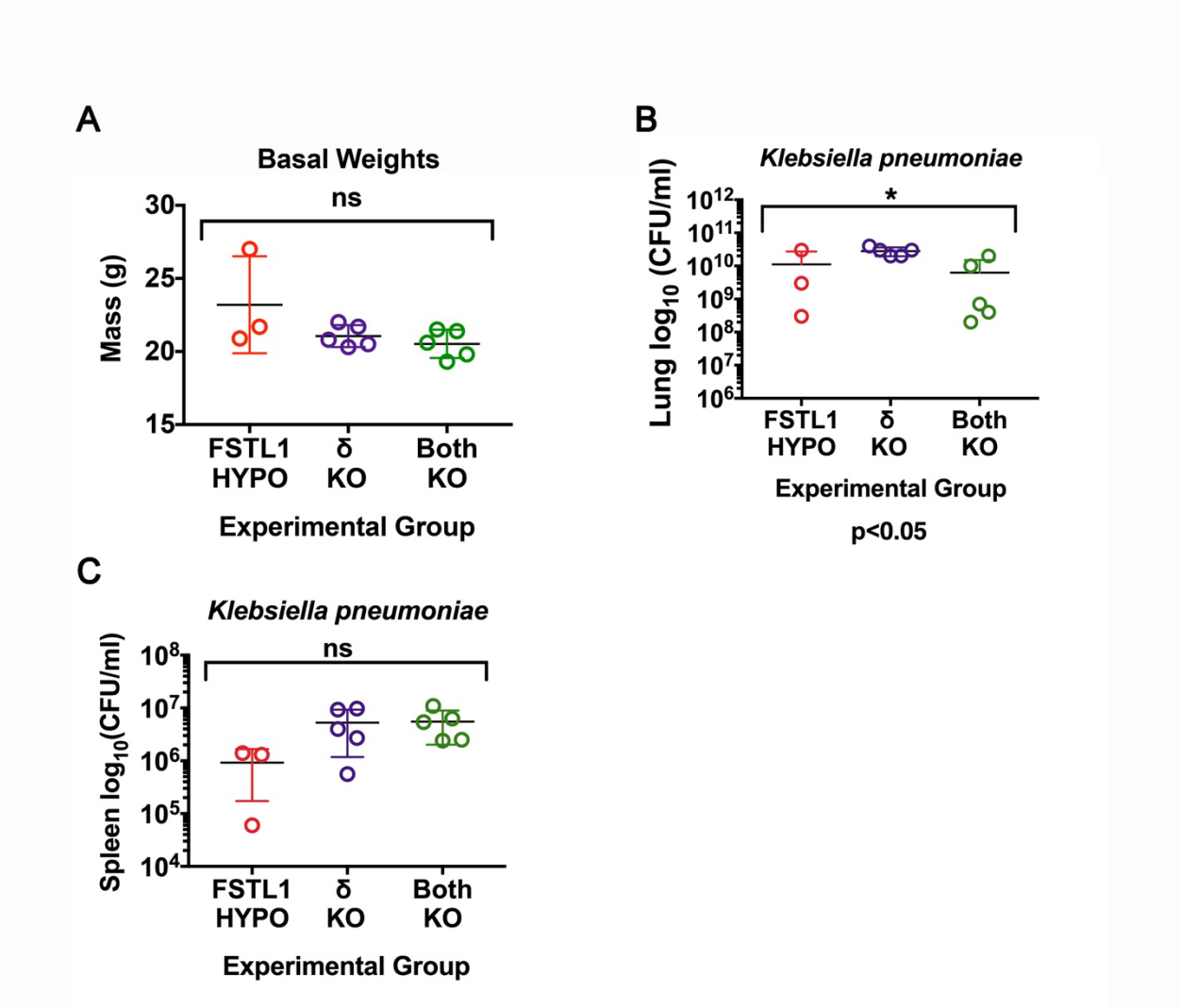

Figure 4. *K. pneumoniae* infection in δ Knockout Models

FIGURE 4. Knocking out gamma delta T cell knockout mice have increased Klebsiella pneumoniae lung burden compared to FSTL1 hypomorphic, gamma delta T cell knockouts and FSTL1 hypomorphic mice after 48 hours of Klebsiella pneumoniae pulmonary infection. **(A)** The basal weights are not significantly different (p=0.1295) among all the experimental mouse groups when analyzed by one-way ANOVA. **(B)** Lung Log10 CFU/ml after 48 hours of Klebsiella pneumoniae infection was significantly different (p=0.0230) when compared using one-way ANOVA analysis. **(C)** Spleen Log10 CFU/ml after 48 hours of Klebsiella pneumoniae infection was not significantly different (p=0.1879) when compared among all experimental groups using one-way ANOVA.

5.3 *K. pneumoniae* Infection in Fstl1 Conditional Knockouts

We next asked the question: Is our phenotype phenocopied in an FSTL1 Conditional Knockout model? Using Rosa26Cre / FSTL1 conditional knockout mice, we addressed whether K. pneumoniae lung bacterial burden would be lower when animals experienced suppression of FSTL1 production during the adult life stage and during infection. This FSTL1 experimental model should not include any developmental effects due to diminished FSTL1 production, in contrast to the FSTL1 hypomorphic mouse model. The FSTL1 hypomophic phenotype was not phenocopied in the FSTL1 conditional knockout model. FSTL1 conditional knockout mice are not protected against Klebsiella pneumonia pulmonary infection after 48 hours (**5C)** after infection but have significant spleen dissemination differences (**5D)**.

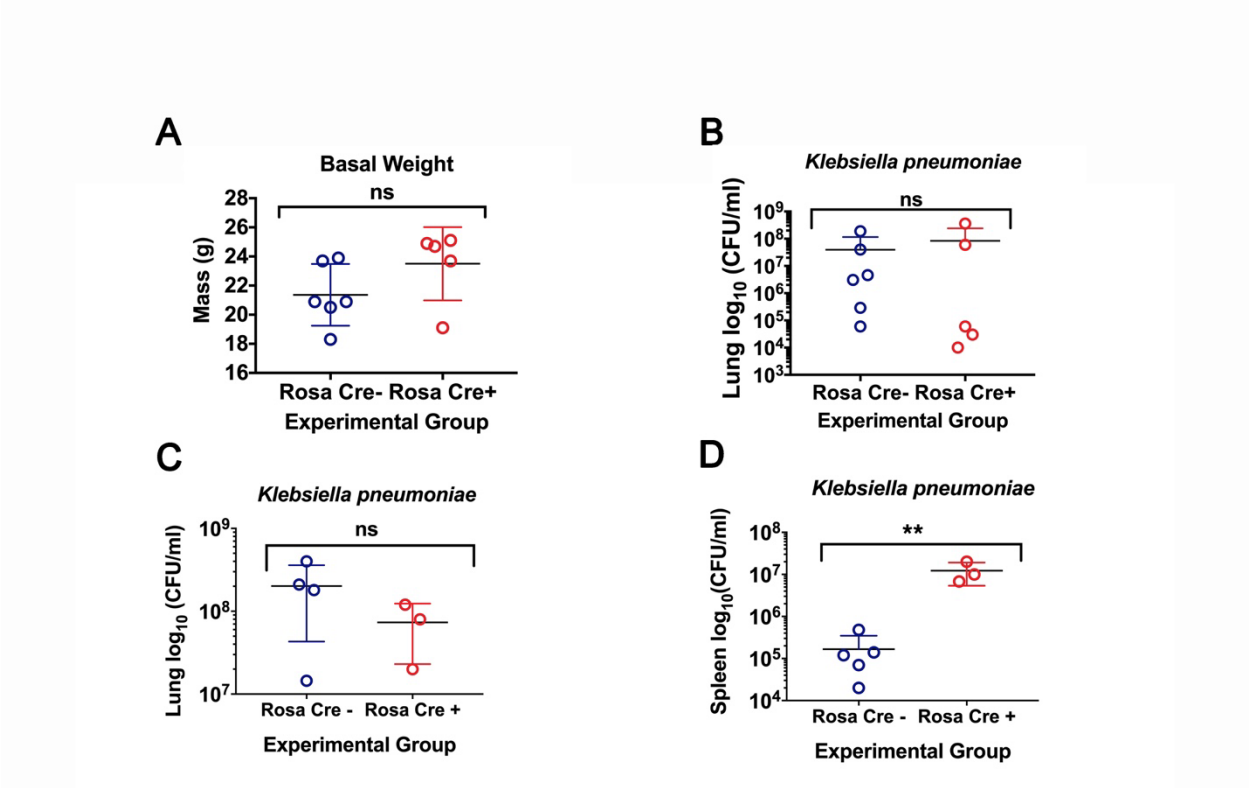

Figure 5. *K. pneumoniae* in FSTL1 Conditional Knockout Model

FIGURE 5. FSTL1 conditional knockout mice are not protected against *Klebsiella pneumonia* pulmonary infection at 24 or 48 hours after infection**. (A)** Conditional knockout and control mice show no significant weight differences (p=0.1607) between groups when analyzed by unpaired Student's T-test. **(B)** Lung Log10 CFU/ml at 24 hours of Klebsiella pneumoniae infection was not significantly different (p=0.3889) when analyzed by unpaired Student's T-test. **(C)** Lung Log10 CFU/ml after 48 hours of Klebsiella pneumoniae infection was not significantly different (p=0 0.2431) between FSTL1 conditional knockouts and control mice when analyzed by unpaired Student's T-test. **(D)** FSTL1 conditional knockouts and controls showed significant differences between Spleen Log10 CFU/ml after 48 hours of Klebsiella pneumoniae infection (p= 0.0059) when analyzed by unpaired Student's T-test.

5.4 *K. pneumoniae* Infection in Neutralizing Antibody Model

We next addressed the question: Is our FSTL1 Hypomorph phenotype phenocopied in an FSTL1 neutralizing antibody model? C57B6 wild-type mice were treated with 200ug anti-FSTL1 antibody or Rabbit IgG control in PBS by intratracheal distillation and intrapleural injection 4 hours post Klebsiella pneumoniae infection start and on morning of day 2. Neutralizing FSTL1 antibody treated mice before and during Klebsiella pneumoniae infection does not confer protection. Therefore, the FSTL1 hypmorph protection phenotype is not phenocopied in the FSTL1 conditional knockout model or the FSTL1 neutralizing antibody model.

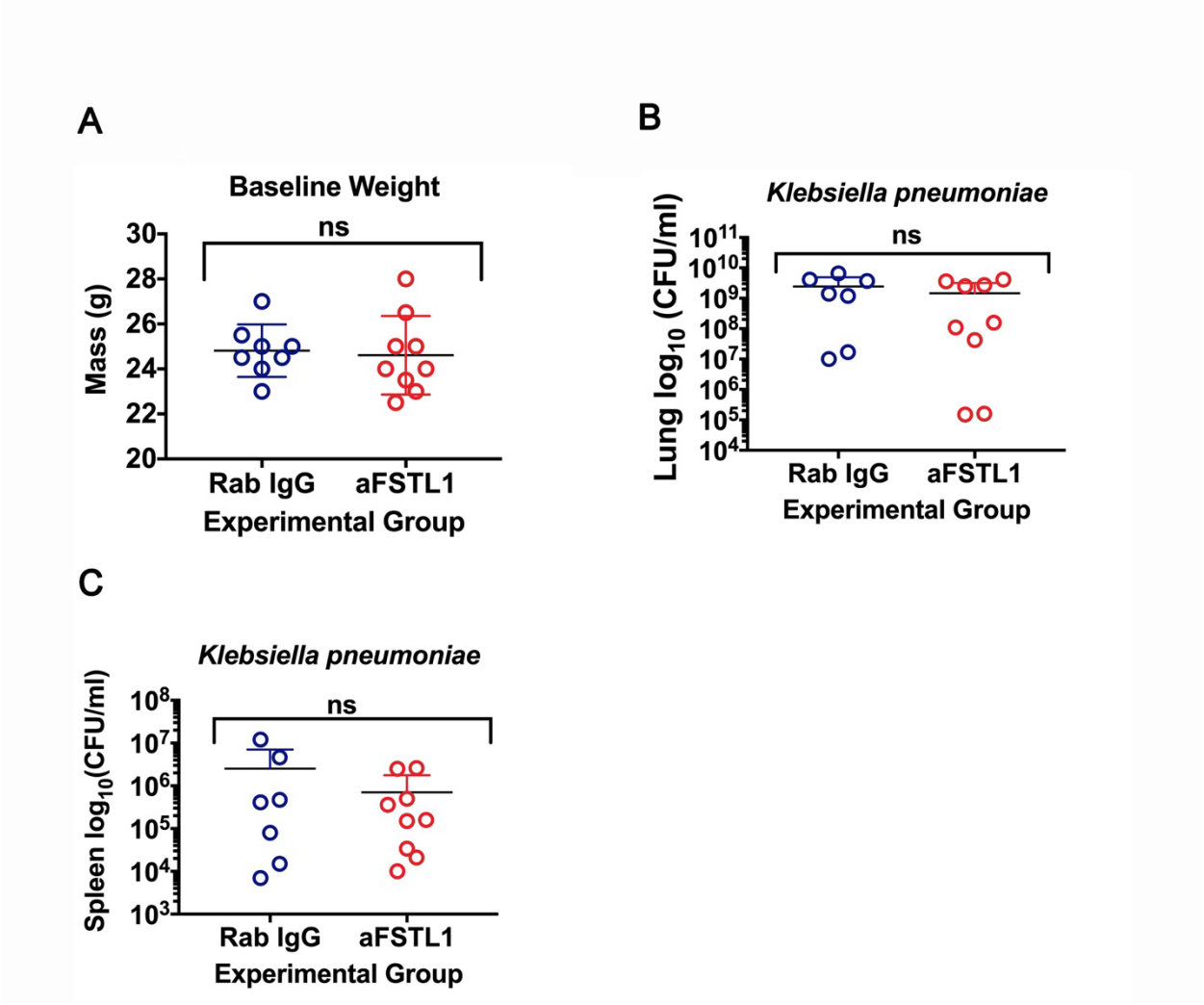

Figure 6. FSTL1 Neutralizing Antibody Model

FIGURE 6. Neutralizing FSTL1 antibody treated mice before and during Klebsiella pneumoniae infection does not confer protection. C57B6 wild-type mice were treated with 200ug anti-FSTL1 antibody or Rabbit IgG control in PBS by intratracheal distillation and intrapleural injection 4 hours post Klebsiella pneumoniae infection start and on morning of day 2. **(A)** Basal weights between the two groups were not significantly different (p=07864), when analyzed by unpaired Student's T-test. **(B)** Lung Log10 CFU/ml was not significantly different (p=0.3708) between the two experimental groups, Rabbit IgG or anti-FSTL1 antibody treatment. **(C)** Spleen dissemination was measured by Spleen Log10 CFU/ml and was not significantly different (p=0.2591) between the two experimental groups, Rabbit IgG or anti-FSTL1 antibody treatment. Results were produced using unpaired Student's T-test.

5.5 *K. pneumoniae* Infection in Recombinant Protein Model

Finally, we addressed the question: If we add FSTL1 protein into the system, will be get the reverse phenotype (higher bacterial burden)? C57B6 wild-type mice were treated with 400ng recombinant FSTL1 protein or bovine serum albumin by intratracheal distillation and intrapleural injection 4 hours post Klebsiella pneumoniae infection start, 24 hours into infection, and 36 hours into 48-hour infection. Daily FSTL1 recombinant protein treatment trends towards conferring Klebsiella pneumoniae protection at 24 hours of infection **(7A)**. Daily FSTL1 recombinant protein treatment trends towards conferring Klebsiella pneumoniae protection resolves after 48 hours **(7D)**.

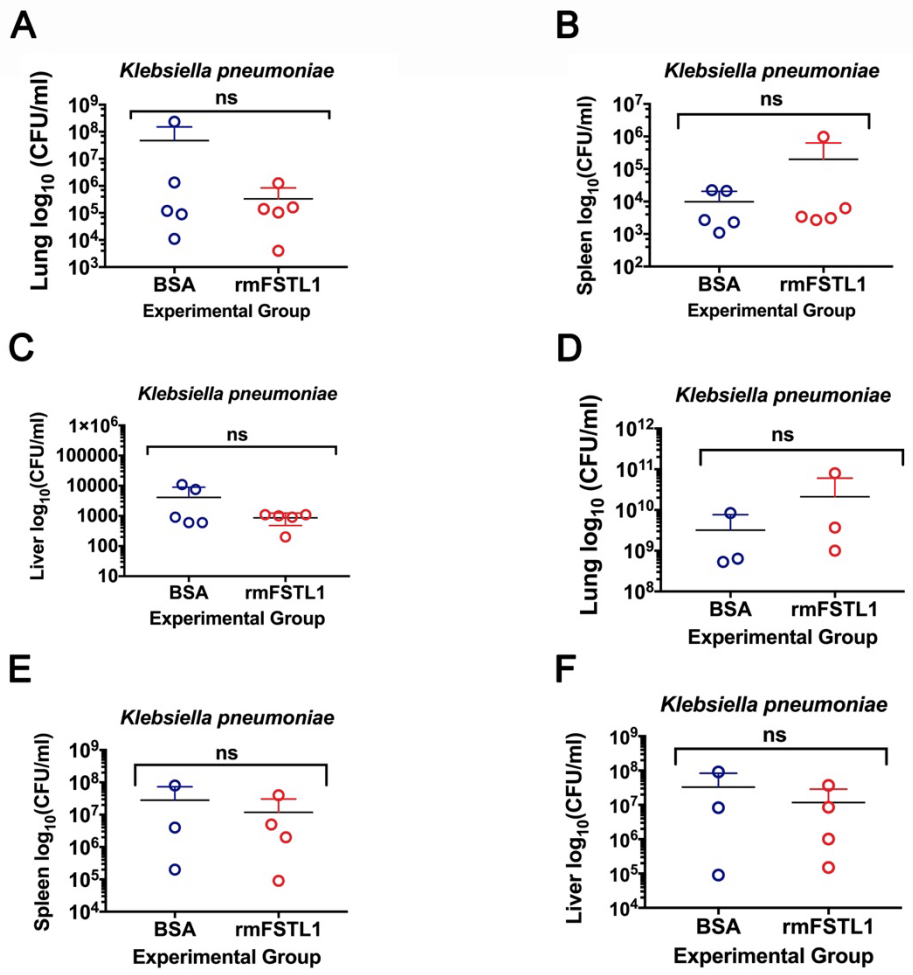

Figure 7. FSTL1 Recombinant Protein Model

FIGURE 7. Daily FSTL1 recombinant protein treatment trends towards conferring Klebisella pneumoniae protection at 24 hours of infection and resolves after 48 hours.
C57B6 wild-type mice were treated with 400ng recombinant FSTL1 protein or bovine serum albumin by intratracheal distillation and intrapleural injection 4 hours post Klebsiella pneumoniae infection start, 24 hours into infection, and 36 hours into 48 hour infection.
(A) 24 hours into Klebsiella pneumoniae infection, Lung Log10 CFU/ml were not statistically significant (p=0.3460) between the BSA and recombinant protein treatment groups. There is a trend for the recombinant FSTL1 treated group to have lower lung burden. **(B)** Spleen CFUs were not statistically significant (p=0.3611) after 24 hours of infection, when analyzed by unpaired Student's T-test. **(C)** Liver CFUs were not statistically significant (p=0.17260) after 24 hours of infection, when analyzed by unpaired Student's T-test. There is a trend for the recombinant FSTL1 protein treated experimental group to have decreased bacterial lung burden. **(D)** 48 hours into Klebsiella pneumoniae infection, Lung Log10 CFU/ml were not statistically significant (p=0.4748) between the BSA and recombinant protein treatment groups. **(E)** Spleen CFUs were not statistically significant (p=0.5348) after 48 hours of infection, when analyzed by

unpaired Student's T-test. **(F)** Liver CFUs were not statistically significant (p=0.4525) after 48 hours of infection, when analyzed by unpaired Student's T-test.

6.0 Discussion

There were several key findings from the results of this series of experiments. FSTL1 hypomorphic mice are protected from *Klebsiella pneumoniae* lung burden after 48 hours with an initial starting inoculum of $1x10^4$ CFU. The FSTL1 hypomorphic mice may have decreased bacterial lung burden in response to an increased magnitude of specific cell types - granulocytes such as neutrophils, or different subtypes of macrophages. Further analysis allowed for clarification that FSTL1 hypomorphic mice have increased genetic expression of *il6, csf3, il1b,* and *il17a.* IL-17A cytokine has been known to act in a pro-inflammatory way by inducing granulocyte colony-stimulating factor. Granulocyte colony-stimulating factor stimulates the proliferation of neutrophils (Hirai source), which may be clearing the *K. pneumoniae* infection, at least partially.

FSTL1 hypomorphic mice also have increased *trdc* and *trdv4* in the lung at 24 hours of infection, implying that γδ T cells are increased during Klebsiella pneumoniae infection in the FSTL1 hypomorphic mouse model. Additional flow cytometry analysis clarified that these γδ T cells produce IL-17A at significantly higher levels than wild-type mice during infection.

Using the global delta knockout mouse model, FSTL1 hypomorphic / delta knockout model, and FSTL1 hypomorphic model we further clarified that γδ Tcells may interact with FSTL1 protein because all three mouse models had significantly different *Klebsiella pneumoniae* lung burden after 48 hours of infection.

Klebsiella pneumoniae protection is not recapitulated in Rosa26Cre/FSTL1 knockout model or neutralizing antibody model at 48 hours of infection. Therefore, the FSTL1 hypomorphic model may depend on features of development that effect *Klebsiella pneumoniae*

infection severity and clearance that are not phenocopied into these other models. The recombinant FSTL1 protein model shows a trend towards lower burden after 24 hours and higher burden after 48 hours of *Klebsiella pneumoniae* infection.

The results from this study bring into question whether the role that FSTL1 plays with BMP signaling where which is responsible for the lower lung bacterial burden during K. pneumoniae infection. The inability to phenocopy the FSTL1 hypomorph phenotype, a lower level of bacterial lung burden after 48 hours, in the other FSTL1 experimental models may be the result of the FSTL1 hypomoprhic mice having reduced FSTL1 protein levels during development, as well as infection. Additional experimentation, *in vivo* and *in vitro*, will have to be conducted to understand the extent of FSTL1's role during *K. pneumoniae* infection.

7.0 Future Directions

Understanding and clarifying the relationship between FSTL1 interactions with specific immune cell mediators will need further experimentation using the mouse models described in this thesis and additional *in vitro* human cell line experiments. The results from my experiments bring up several additional questions about the mouse models. One such question is whether FSTL1 protein interventions are only short-lived and masked by longer infection endpoints? The neutralizing antibody model and FSTL1 conditional knockout models were both unable to phenocopy the FSTL1 hypomorphic model. These models may not have been able to phenocopy the lower lung burden phenotype due to improper antibody dosing for the neutralizing antibody model or improper tamoxifen dosing frequency for the conditional knockout model. Additionally, the science behind the FSTL1 hypomorphic model explains that these mice produce around 60% FSTL1 protein, so FSTL1 protein is not completely cleared from the system. Therefore, the lower lung burden may be the result of an intermediate production amount of FSTL1 protein, unlike the idea that FSTL1 protein is completely out of the pulmonary system in the neutralizing antibody model or conditional knockout models. Additionally, the FSTL1 hypomorphic mice may have some FSTL1 producers from the bone marrow or other bodily organs that travel to the lung site of infection. Homeostatic mechanisms may be at play where FSTL1 at 60% is able to influence other cell types and result in decreased lung burden. Additional experimentation must be completed in order to answer these questions.

The results from these experiments bring up the question: Are there human individuals that produce a similar amount of FSTL1 protein in the lung to the FSTL1 hypomorphic mice? If these individuals are susceptible to *Klebsiella pneumoniae pulmonary* infection, do they have

lower bacterial lung burden anytime during infection? In other words, are the results from the FSTL1 hypomorphic model translational for the human population?

If the above individuals exist in the population, should we identify them via genetic testing? If we were to develop an intervention based on genetics, what would requirements would we need for genetic test to be actionable? The core principles of public health genetics call for an FSTL1 genetic test to meet the criteria of treatment availability, incidence, clear benefits, and feasibility. I would ask if there is effective treatment for all screened, with early treatment being better than late treatment. We would need human clinical data of *Klebsiella pneumoniae* infection in order to answer this requirement. For the incidence criteria, we would need to address whether *Klebsiella pneumoniae* pulmonary infection has a high incidence in the population. In terms of hospital settings, Klebsiella pneumoniae is the third most common cause of nosocomial infections so I believe that these criteria would be met. For the clear benefits criteria, we need to ask whether there would be clear benefits for the genetic test. Currently, there are no FSTL1 based therapies or interventions, so there are not clear benefits to genetic testing for FSTL1 production genotypes. The last criteria of feasibility would not currently be met as well because there are no data on the costs for FSTL1 genetic testing. Therefore, genetic testing is not applicable at this point in time without further human clinical data. Further clinical research must be done to address the translational aspects of this project and possible public health interventions.

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