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Regulation and Role of Chitotriosidase during Lung Infection with *Klebsiella pneumoniae*

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Abstract

Chitinases and chitinase-like proteins are an evolutionary conserved group of proteins. In the absence of chitin synthesis in mammals, the conserved presence of chitinases suggest their roles in physiology and immunity, but experimental evidence to prove these roles are scarce. Chitotriosidase (chit1) is one of the two true chitinases present in mammals and the most prevalent chitinase in humans. Here, we investigated the regulation and the role of chit1 in a mouse model of *Klebsiella pneumoniae* (Kp) lung infection. We show that chitinase activity in bronchoalveolar lavage fluid (BAL) is significantly reduced during Kp lung infection. This reduced activity is inversely correlated with the number of neutrophils. Further, instilling neutrophil lysates in lungs decreased chitinase activity. We observed degradation of chit1 by neutrophil proteases. In a mouse model, chit1 deficiency provided a significant advantage to the host during Kp lung infection by limiting bacterial dissemination. This phenotype was independent of inflammatory changes in chit1$^{-/-}$ mice as they exerted a similar inflammatory response. The decreased dissemination resulted in improved survival in chit1$^{-/-}$ mice infected with Kp in the presence or absence of antibiotic therapy. The beneficial effects of chit1 deficiency were associated with altered Akt activation in the lungs. Chit1$^{-/-}$ mice induced a more robust Akt activation after infection. The role of the Akt pathway in Kp lung infection was confirmed by using an Akt inhibitor, which impaired health and survival. These data suggest a detrimental role of chit1 in Kp lung infections.

Introduction

Lung infections are the 8th leading cause of death in the United States (CDC, 2017). Pneumonia due to bacterial pathogens is a major clinical challenge. Due to anatomical and physiological reasons, lungs are constantly exposed to microbial agents. To deal with invading microbes, lungs are well equipped with various host defense mechanisms (1, 2).

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However, opportunistic pathogens often overcome these host defense mechanisms, especially in persons with an impaired host defense such as those in hospitals with underlying diseases, or those on ventilators (3). *Klebsiella pneumoniae* (Kp) is one such opportunistic gram-negative bacterium (4). Pulmonary bacterial infections due to Kp result in substantial mortality and therapeutic costs and Kp is the 3rd leading cause of hospital-acquired bacterial pneumonia (4, 5). Significant increase in mortality is observed when infections spread to the peripheral organs, which can lead to sepsis and septic shock. Mortality due to Kp bacteremia can be as high as 50–70 % (6, 7). Although antibiotics are the main therapeutic intervention used, therapeutic failure is common and significant mortalities still persist (8). The emergence of multi-drug resistant (MDR) strains of this pathogen, especially strains that produce carbapenamase, are putting further limitations on the currently available therapies (9). While the discovery rate of new antibiotics has been dismal (10), exploring host mechanisms that contribute to the regulation of infection could help to develop new therapies. Further, there is always a risk of emerging resistant strains against newly developed antibiotics. On the other hand, host-targeted therapies are not prone to develop resistance by pathogens, at least theoretically. However, very little is known about the mechanisms involved in bacterial dissemination from the lung to systemic circulation and the host factors that are responsible for this phenomenon.

Chitinase and chitinase-like proteins (CLPs) are a conserved group of proteins that belong to the 18-glycosyl hydrolase family (11). In the absence of chitin biosynthesis in mammals, chitinases were hypothesized to have important roles in physiology. Their well-documented regulation in various diseases and pathological conditions further support their important biological roles (12–14). Chitotriosidase (chit1) is one of the two true chitinases present in mammals, the other one being Acidic mammalian chitinase (AMCase) (15, 16). While chit1 is the most prominent chitinase present in humans, mice express both chit1 and AMCase (17). The role of chit1 in fungal and parasitic infection is indicated by both epidemiological and experimental studies (18–21). A significant proportion of various populations including Asians, Caucasians and Indians are deficient (5–20%) in enzymatic activity mainly due to a 24-bp duplication mutation (22, 23). Interestingly, these individuals without chitinase activity do not express any obvious related phenotypes or reported abnormalities. However, how these individuals respond to bacterial infections is not known.

The role of chit1 has been proposed in host immunity mainly due to its high expressions at anatomical locations where host pathogen interactions take place, such as the lungs and the gut (17, 24). Further, supporting this belief, chit1 is stored in and released from macrophages, one of the main effector cells against invading pathogens (25). However, the regulation and role of chit1 in bacterial infection has not been explored *in vivo.*

In the current study, we explored the regulation and the role of chit1 in Kp lung infection. Here we report that chitinase activity is significantly down-regulated during Kp infection, which is mediated by the degradation of chit1 by neutrophil elastases. In this infection model, we show that chit1 deficiency provides a significant advantage to the mice by limiting bacterial dissemination. This resulted in improved survival in mice in the presence or absence of antibiotic therapy. Altered Akt activation in chit1-deficient mice was associated with improved outcomes in Kp infection.

Material and Methods

Animal studies

All animal studies were done according to IUCAC approved protocols at Yale University.

Bacterial infection model

Mouse adapted laboratory strain of *Klebsiella pneumoniae* (Kp) ATCC 43186 was grown on LB plates from the glycerol stocks stored in −80 C. Next day, a single colony was transferred to liquid broth to culture overnight and next day it was sub cultured for 1 h to bring the bacteria into log phase of growth. The numbers of CFUs were estimated by measuring OD at 600 nm and were confirmed by plating the inoculum. Mice were inoculated intratracheally by injecting 50 μl of PBS solution containing 5000 CFUs of Kp.

For intratracheal inoculation, mice were anaesthetized using a mixture of ketamine and xylazine (100 and 10 mg/kg respectively). A small incision was made on the neck to expose the trachea, and bacterial suspension was instilled directly into the trachea. The wound was sealed with Vetbond surgical glue, and the mice were observed until they recovered from anesthesia.

For peritoneal infection, 10^4 CFUs of Kp, suspended in 200 μl of PBS, were injected into the peritoneal cavity of mice. Mice were euthanized at 24 h post infection to harvest peritoneal lavage fluid, spleen, and BAL. Peritoneum was lavaged twice with 5 ml sterile PBS each time.

For Pseudomonas aeruginosa infections, PAO1 strain was grown in the similar manner as described for Kp. Mice were inoculated intratracheally with 1×10^7 CFUs per mouse and organs were harvested at 12 hours post infection.

For LPS administration in the lungs, LPS from Pseudomonas aeruginosa or from Kp (Sigma Aldrich, St. Louis, MO) were used at 5 μg/mouse by intratracheal route and mice were sacrificed at 12 hours post infection.

Purification of neutrophils and preparation of neutrophil lysates

Mouse bone marrows were isolated from donor mice and flushed to isolate bone marrow cells. Purified neutrophils were isolated using a neutrophil isolation kit from Stem cell technologies according to the manufacture's protocol. This kit utilizes a negative selection method using magnetic beads.

Measurement of chitinase activity

Chitinase activity in the BAL samples was measured using a fluorescence-based assay. BAL samples (5μl) were incubated with 4-Methylumbelliferyl β-D-N,N′-diacetylchitobioside hydrate solution for 15 minutes at 37 °C and the fluorescence intensity was measured at 450 nm. BAL samples from IL-13 transgenic mice were used as positive controls as previous studies have indicated significant elevation of chitinase activity in these mice (26).

Western blot studies

Western blots of BAL samples were performed by loading 30 μl of BAL samples from each mouse in 4–20% gradient SDS page gel. For lung tissue and cell lysates, 30 μg and 10 μg of protein, respectively, were loaded. After electrophoresis, the gels were transferred to a PVDF membrane using the Trans-Blot system from Biorad. Membranes were then blocked with 5% milk for 1 h and incubated with primary antibodies overnight. HRP labeled secondary antibodies were used. Bands were detected by using HRP substrate. Band intensities were measured using the BioRad chemiDoc MP imaging system from BioRad. Antibodies to Phospho-Akt (T308), total Akt, and anti-rabbit secondary antibodies were purchased from Cell Signaling Danvers, MA. Antibodies to Chit1 and AMCase were purchased from LS Bio, Seattle, WA. β-actin was purchased from Santa Cruz Biotechnologies.

Quantitative PCR

Total RNA from the lung tissue was extracted using the Qiagen RNAeasy Kit as per manufacturer's instruction. cDNA was synthesized using the iScript reverse transcriptase kit using instructions provided with the kit. qPCR assays were performed using the syber green mastermix. Following primers were used in this study:

AMCase

F- ACA AGC ATC TCT TCA CTG TCC TGG T

R- TGG ATG TTG GAA ATC CCA CCA GCT

Chit1

F- CGG CAG GAA CTA AAT CTT CCA T

R- TGG GCG TGG CTC AGG TAT

18S

F- -GCA ATT ATT CCC CAT GAA CG

R- AGG GCC TCA CTA AAC CAT CC

Harvesting BAL samples

Mice were euthanized at a given point of time post infection or at baseline as indicated. The trachea was exposed by making a small cut on the neck and then inserted with a 22 G catheter as described before (27). Lungs were lavaged by two aliquots of 750 μl of ice-cold sterile PBS. BAL was kept on ice or 4 °C until further processing. These BAL samples were centrifuged to pellet the cells and the cell free supernatant was collected in separate tubes and stored at −80 °C for further analysis.

Enumeration of bacterial burden in BAL, Lung and Spleen

Small aliquots of BAL samples were serially diluted in sterile broth and plated on agar plates. Aseptically isolated left lung or spleens were homogenized in 1 ml of sterile PBS and then serially diluted and plated on agar plates. Numbers of CFUs were estimated by counting the number of colonies on the agar plates after overnight incubation at 37 °C.

Cell count and differential counts

Total numbers of WBCs in the BAL samples were collected by re-suspending the cell pellet obtained from BAL samples from mice into PBS. The cells were counted using the Beckman Coulter cell counter. Approximately 1×10^5 cells were used to prepare cytospin slides, which were stained with HEMA-3 stain, and the number of macrophages and neutrophils were estimated by counting at least 200 cells per slide.

Lung Pathology Scoring

Lungs were inflated with 0.5% low melting agar and fixed in formalin. Tissue sections were stained with hematoxylin and eosin. Lung scoring was done based on scores from 0 to 4, where 0 is no pathology while 4 is severe. Scores for peri-bronchial and peri-vascular inflammation were added for each mouse.

ELISA and Bio-plex assays

Cytokines in the BAL samples were measured using conventional sandwich ELISA duoset kits from R & D as per manufacturer's instruction. Briefly, 96-well plates were coated overnight with capture antibody. Unbound antibody was washed off the next day and the test samples were loaded and incubated for 2 h. Detection antibody was added after washing off the samples. Detection antibody was washed off and streptavidin HRP was added to incubate for 20 minutes. After washing off unbound streptavidin, TMB substrate was added to react with bound HRP for 20 minutes. Stop solution was added to stop the reaction and absorbance was measured at 450 nm. The Bioplex cytokine assay was performed using Bio-Plex Pro™ Mouse Cytokine kit from Bio-Rad according to the instruction provided with the kit.

Survival studies

For survival studies, mice were infected with 5×10^3 CFUs of Kp and were observed every day for mortality. Mice were euthanized once they were considered humane concern and considered as dead. Blood samples were collected from some of these mice, from orbital sinus while under anesthesia by ketamine/xylazine at given time points to estimate bacterial burden in the blood. For survival with antibiotics, two doses of antibiotics were administered at 48 and 60 h post infection.

Primary macrophage and cell line culture

Mouse bone marrow derived macrophages from wild type and chit1$^{-/-}$ mice were developed by culturing the bone marrow in RPMI containing 10% fetal bovine serum and 20 % of L929 cell conditioned media, for 7 days. Cells were treated with *Kp* LPS at 500 ng/ml for

the indicated time points. RAW 264.7 cells from ATCC were grown in DMEM with 10% FBS and 1% penicillin streptomycin solution.

Statistics

Data were analyzed using graph pad prism software version 7. Two groups were compared using student's t test. Experiments comprising more than two groups were analyzed using one way ANOVA and Sidak's multiple comparison test was used. For survival studies, Kaplan Meier curves were prepared and data were analyzed using Wilcoxon test.

Results

Airway chitinase activity is down-regulated during infection and inflammation

Chitinase activity is dynamically regulated during various inflammatory conditions and is believed to be an important mediator of inflammation and disease progression (12, 16, 28). To understand the regulation of chitinase activity during bacterial lung infection, chitinase activity was measured in the BAL samples of mice infected with Kp for different durations. At baseline, mice have a marked chitinase activity which decreases in a time dependent fashion during Kp infection and by 48 h post infection, a significant drop in chitinase activity was observed (596 ± 43 vs 390 ± 33 AU, $P < 0.05$, Fig 1 A). A similar drop in the chitinase activity was observed during Pseudomonas infection or sterile inflammation with the bacterial product LPS (394 ± 32 vs 223 ± 26 AU, $P < 0.01$, Fig. 1 B). Unlike BAL samples, no detectable chitinase activity was observed in the peritoneal lavage fluids obtained from mice (Sup.Fig. 2C). To determine whether infection with Kp leads to the down-regulation of the expression of chitinases, the mRNA levels of chitotriosidase and AMCase were measured. Expression levels of chit1 (1.00± 0.42 vs 0.94 ± 0.43, P = ns, Fig. 1 C) and AMCase (1.00 ± 0.40 vs 1.03 ± 0.46, P = ns, Fig. 1 D) in the lungs of infected mice were similar to that of uninfected mice suggesting that the chitinase expression is not responsible for the decreased activity during infection. Together, our results suggest that chitinase activity is down-regulated during bacterial infection and LPS-mediated inflammation without altering the lung expression of true chitinases.

Inflammatory cells mediate down-regulation of chitinase activity during lung infection by cleaving chitotriosidase

Up-regulation of chitinase activity is often associated with the type 2 inflammatory responses, especially those mediated by eosinophils (14, 26). However, the role of inflammatory cells such as neutrophils in regulating chitinase activity is not known. We found that neutrophil infiltration in the lung during Kp infection (Fig. 2A) inversely correlated with chitinase activity in the BAL of mice ($R = -0.93$, $P < 0.05$, Fig 2B). No significant correlation was observed with macrophage infiltration and chitinase activity ($R = -0.84$, P = ns, Fig. 2D). To establish a causal relationship between neutrophils and down-regulation of chitinase activity, we instilled neutrophil lysates directly to the lungs of mice and measured chitinase activity into the BAL samples of mice. Similar to infection and inflammation, instillation of neutrophil lysates resulted in a significant decrease in chitinase activity in the BAL samples (800 ± 45 vs 601 ± 39 AU, $P < 0.05$, Fig. 2E). By western blot analysis, we observed a significant reduction in the levels of chit1 protein during infection as

well as upon instillation of neutrophil lysates (Fig. 2 F & G). The levels of AMCase did not change in the BAL samples after infection or after instillation of neutrophil lysate. In agreement with these results, we also observed that the treatment of BAL fluid with neutrophil elastase significantly degrades chit1 but no susceptibility of AMCase to elastase was observed (Fig. 2 H). Together these results suggest that neutrophil infiltration during lung infection or sterile inflammation contribute toward decreased chitinase activity mediated by proteolytic cleavage of chitotriosidase.

Chit1 deficiency in mice limits bacterial dissemination and weight loss during *Klebsiella* infection

Chit1 is the most prominent chitinase present in humans and also contributes significantly to chitinase activity in mice (17) and Sup Fig. 2). To understand the role of chitinases during lung infection, we infected chit1 deficient (chit1$^{-/-}$) mice to study their weight loss, lung bacterial burden, spleen bacterial burden (as a marker of dissemination), and total protein content in BAL (as a marker of lung injury). These data show that at 48 h post infection, chit1$^{-/-}$ mice maintain their weight closer to baseline compared to wild type mice (Weight loss in wild type 14.64 ± 0.84% vs 12.37 ± 0.74 % in chit1$^{-/-}$ mice, P = 0.05, Fig. 3A). The decreased weight loss corresponded to decreased bacterial burden in spleen of chit1$^{-/-}$ mice by a log change (6.06 ± 0.26 in wild type vs 5.05 ± 0.25 Log CFUs/Spleen in chit1$^{-/-}$ mice, P < 0.01 Fig. 3B), while similar bacterial burden in the BAL was observed (6.20 ± 0.18 in wild type vs 5.97 ± 0.17 Log CFUs/ml BAL in chit1$^{-/-}$ mice, P = ns, Fig. 3C). Similar to BAL, the bacterial burden in the lung tissue were not different among the two groups (5.52 ± 0.45 vs 6.39 ± 0.41, P = ns). No difference in the lung injury was observed as indicated by the total protein content in the BAL (169.50 ± 13.25 in wild type vs 186.80 ± 12.95 μg/ ml BAL in chit1$^{-/-}$ mice, P = ns, Fig. 3D). In contrast, upon peritoneal infection, similar bacterial burdens were observed in the peritoneal lavage fluid, BAL, and spleen in wild type and chit1$^{-/-}$ mice (Sup Fig. 3B, C and D). Similar weight loss between wild type and chit1$^{-/-}$ mice was observed during peritoneal infection (Sup Fig. 3A), along with similar levels of inflammation (Sup Fig. 3E and F), suggesting lung specific protective effects of chit1 deficiency during Kp infection. Interestingly, unlike BAL fluid, which has significant chitinase activity at baseline, the peritoneal lavage fluid had no detectable chitinase activity (Sup. Fig. 2C)

To test whether these protective effects are specific to Kp or extends to other gram negative infections, we use a Pseudomonas infection mouse model. Using this mouse model, we did not see any protection in Chit1 −/− mice as evident by increased bacterial load in lung tissues as well as in the spleen (Sup Fig. 3G, H and I). Together, these results suggest that chit1 specifically regulates bacterial dissemination during Kp lung infection without significantly altering pulmonary bacterial clearance and lung injury.

Chit1$^{-/-}$ mice have similar inflammatory responses during Kp lung infection

To understand the possible mechanisms behind improved health and limited bacterial dissemination in the absence of chit1, we sought to determine the inflammatory response during lung infection with Kp. Mice infected with Kp leads to the elevation of many of the inflammatory cytokines resulting in inflammatory cell recruitment (data not shown).

Compared to wild type mice, chit1$^{-/-}$ mice had similar inflammatory cell infiltration at 48 h after Kp infection (0.55 ± 0.07 in wild type vs 0.57 ± 0.09 × 10^6 / mouse in chit1$^{-/-}$ mice, P = ns, Fig. 4A). The number of neutrophils (0.32 ± 0.07 in wild type vs 0.28 ± 0.06 × 10^6 / mouse in chit1$^{-/-}$ mice, P = ns, Fig. 4B) and macrophages (0.19 ± 0.02 in wild type vs 0.27 ± 0.04 × 10^6 /mouse in chit1$^{-/-}$ mice, P = ns, Fig. 4C) were similar in these mice BALs after lung infection. Histological analysis revealed cell infiltration and consolidation in the lung tissue of infected mice but there was no apparent difference between lungs from wild type and chit1$^{-/-}$ mice as indicated by lung pathology scores (4.73 ± 0.59 in wild type vs 4.9 ± 0.57 in chit1$^{-/-}$ mice, P = ns, Fig. 4 K & L). The levels of cytokines were also measured in the BAL samples obtained from the infected mice. The levels of various inflammatory cytokines such as TNFα, IL-β, IL-6, IL-12, IFNγ and IL-17 were not different between the two groups upon infection (Fig. 4 D-I). Similarly, the anti-inflammatory cytokine IL-10 was similar between infected wild type and chit1$^{-/-}$ mice (Fig. 4J). The levels of other tested cytokines such as IL-2, IL-4 and IL-5 were below the detection limit (1pg/ml) in BAL samples of both wild type and chit1$^{-/-}$ mice. Corresponding to these results, bone marrow derived macrophages obtained from wild type and chit1$^{-/-}$ mice had similar cytokine responses when stimulated with LPS (Sup Fig. 4 D & E). Also, macrophages cell line RAW 264.7 cells produced similar cytokines in response to LPS in the presence or absence of recombinant chit1 (Sup Fig. 4 F & G). Interestingly, BMDMs obtained from chit1−/− mice had better control on bacterial growth in-vitro compared to the BMDMs obtained from wild type mice (Sup Fig. 4 H).

Overall, these results indicate that chit1 deficiency does not play a significant role in regulating inflammatory response during lung infection but still contribute to the protection against Kp infection in macrophages.

Chit1$^{-/-}$ mice regulate early dissemination of bacteria and have a survival advantage with Kp lung infection with or without antibiotic therapy

To better understand the mechanisms of lower bacterial burden in the spleen of chit1$^{-/-}$ mice, we sought to determine the time course of bacterial dissemination in these mice. After infection with Kp, bacterial burden was measured in the blood samples of these mice. Chit1$^{-/-}$ mice have lower bacterial burden in their blood compared to wild type mice, which is evident as early as 24 h after infection and remains higher up to 48 h (196.18 vs 8.82 × 10^3 CFU/ ml at 24 h, 185.40 vs 28.75 × 10^3 CFU/ ml at 36 h and 544.67 vs 23.64 × 10^4 CFU/ ml at 48 h, P = .011), suggesting better control of bacterial dissemination in the chit1$^{-/-}$ mice (Fig. 5A). Next, we investigated if limiting the bacterial dissemination results in better survival in chit1$^{-/-}$ mice. As expected, we observed a significant survival advantage in the chit1$^{-/-}$ mice during lung infection. On day 3, while 70 % of mice succumbed to infection in the wild type group, only 44 % died of infection in the chit1$^{-/-}$ group (Fig. 5B) P < 0.05. To further mimic a clinical situation, we administered two doses of antibiotics in the mice at 48 and 60 h post infection to understand the role of chit1 deficiency in this model. While even after two doses of antibiotics, wild type mice maintained detectable bacterial burden in their circulation; the chit1$^{-/-}$ mice effectively cleared circulating pathogens after similar doses of antibiotics (Fig. 5C). The lower bacterial burden in chit1$^{-/-}$ mice after antibiotic treatment resulted in a rather dramatic increase in the survival during Kp lung infection. While

approximately 80 % of mice survived in the chit1$^{-/-}$ group, only 10 % of mice survived in the wild type group (Fig. 5D) $P < 0.05$. Taken together, these data strongly suggest that chit1 deficiency provides mice with a survival advantage, with or without antibiotics, due to better control of early dissemination.

Altered Akt activation in chit1$^{-/-}$ mice during lung infection

Akt signaling has been shown to play important roles during host pathogen interactions (29). Chitinase-like proteins have been shown to regulate the Akt signaling pathway. AMCase has been shown to protect epithelial cells apoptosis by regulating PI3K-Akt pathway while chitinase-like protein Chil1 directly activates Akt signaling (30, 31). We sought to determine whether chit1 regulates the Akt pathway during Kp infection. Our data indicate that chit1$^{-/-}$ mice maintain a lower Akt activation compared to wild type mice at baseline (1.00 ± 0.14 in wild type mice vs 0.45 ± 0.03 in chit1$^{-/-}$ mice, $P < 0.01$, Fig. 6A). Interestingly, upon Kp lung infection, chit1$^{-/-}$ mice have a significantly higher activation of Akt signaling compared to wild type mice (1.00 ± 0.07 in wild type mice vs 1.27 ± 0.10 in chit1$^{-/-}$ mice, $P < 0.05$ Fig. 6B). Similar to the lungs, bone marrow-derived macrophages from chit1$^{-/-}$ mice had significantly lower activation of Akt at baseline (1.0 in wild type cells vs 0.79 ± 0.04, in chit1$^{-/-}$ cells, $P < 0.05$); however, upon stimulation with LPS, a robust activation of Akt was observed in chit1$^{-/-}$ macrophages (0.79 ± 0.04 at baseline vs 1.14 ± 0.12 with LPS stimulation, $P < 0.005$) but not in wild type macrophages (1.0 at baseline vs 0.96 ± 0.04 with LPS stimulation, P =ns) (Fig. 6C). On the other hand, there were no differences in the activation of the MAPK pathway as measured by the activation of P42/44 or P38 at either baseline or upon infection (Fig. 6D, E, G & H). Similar results were obtained upon in bone marrow derived macrophages stimulation with LPS (Fig. 6F & I).

Inhibition of Akt pathway impairs bacterial clearance, dissemination, and survival in *Klebsiella* lung infection

To further understand the role of Akt during Kp lung infection, we used Akt inhibitor wortmannin. A single dose of wortmannin impaired health (Weight loss 4.78 ± 0.51 in controls vs 9.14 ± 0.65 in treated, $P < 0.005$, Fig. 7A) and decreased survival in mice (Median survival 4 day in controls vs 2 day in treated, $P < 0.01$, Fig. 7B). As expected, the wortmannin treated mice had significantly elevated bacterial load in the spleen (3.21 ± 0.556 vs 7.09 ± 0.30 CFUs/spleen) and BAL (5.46 ± 0.14 vs 6.85± 0.04 CFUs/ml BAL) (Fig. 7C & D, respectively), suggesting the importance of the Akt pathway during Kp lung infection.

Discussion

Lung infections result in significant morbidity and mortality in humans. In the fight against infectious bacteria, antibiotics provided us with a significant edge over pathogens, but this advantage diminished with the development of antibiotic resistance in pathogens (32). Currently, MDR strains are threatening to revert all the advantages achieved in the last eight decades. The rapidly evolving antibiotic resistance, and slow antibiotic discovery prompted us to explore host mechanisms that might boost host immunity to help survive infections.

Chitinases are an evolutionally conserved group of proteins from yeasts, arthropods and fruit flies to mammals including humans (Sup Fig. 1). Shared and conserved areas of the CHIT1 gene can be seen across the different species from the depicted gene tree. The presence of chitinases in humans and other mammals has been puzzling in the absence of chitin synthesis. The obvious role of chitinases in physiology or host defense has not been proven experimentally. Also, a significant human population (5–20% of healthy human population) that is devoid of chitinase activity due to a 24 bp mutation (22, 23, 33). However, a well-documented regulation of chitinases during many diseases and pathological conditions (16, 34, 35), suggest specific regulatory mechanisms of the activity and functions associated with chitinases.

In the current study, we explored the regulation of chitinases during lung infection with *Klebsiella pneumoniae* (Kp), a leading pathogen in hospital-acquired pneumonia and in patients with complex lung diseases (5, 36, 37). Kp infection resulted in a time dependent decrease in chitinases activity in the BAL fluid (Fig. 1A), which was independent of gene expression of AMCase and Chit1 (Fig. 1C & D). The role of live pathogens in decreasing chitinase activity in the infected mice was excluded due to similar down-regulation of chitinase activity in LPS injected mice (Fig. 1B). These data suggest that live pathogens are not essential to decrease chitinasae activity.

To understand the mechanisms underlying the down-regulation of chitinase activity, we characterized the inflammatory response during Kp infection. We observed not only a strong negative correlation between chitinase activity and the number of neutrophils in BAL, but also direct instillation of neutrophil lysates in the lung effectively decreased chitinase activity, establishing a causal role of neutrophils. In our BAL samples from mice that were infected with Kp or instilled with neutrophil lysates, we observed lower levels of chitotriosidase protein, suggesting a mechanism for decreased chitinase activity (Fig 2 F & G). A similar breakdown of chitotriosidase was observed when BAL samples were incubated with neutrophil elastase (Fig. 2H). Supporting our observations, a recent report studying the interaction of neutrophil enzymes with chitotriosidase in fungus infected CF patients reported that neutrophil enzymes can directly cleave chitotriosidase present in the BAL samples of patients with cystic fibrosis (38). Interestingly, we did not see a reduction in the levels of AMCase, the second chitinase present in mammals (Fig. 2 F & G). Supporting this observation, treatment of BAL with neutrophil elastase had no effect on AMCase levels. Overall, we propose here that neutrophils recruited during pulmonary infections directly decrease chitinase activity by cleaving chitotriosidase with minimal effect on AMCase. It is important to note that only airway lavage fluids possessed significant chitinase activity at baseline, which was absent in the peritoneal lavage fluids (Sup Fig. 2 C).

Many pathological conditions and diseases such as Gaucher’s disease, interstitial lung disease, COPD, and diabetes have been shown to be associated with increased chitinase activity (16, 34, 35, 39). However, these studies did not explore the type of inflammation or cell types involved in these pathological conditions, or the role of chit1 as a contributor to the underlying disease or patho-physiological conditions. Also, whether increased chitinase activity contributes to the increased susceptibility to infections in many of these diseases, is not known.

To understand the role of chitinases in lung infections, we used mice that are deficient in chit1. Chit1 is the most prominent chitinase in humans and almost all of chitinase activity can be attributed to it (16). However, AMCase has been reported to be the major chitinase in mice (15, 26). The role of chit1 in mouse lung or its contribution to the overall chitinase activity has not been well appreciated (17). In this report, we present data to show that chit1 is a significant contributor to the chitinases activity in mice, as chit1 knockout mice have significantly lower chitinase activity in BAL and serum (Sup Fig. 2). Emphasizing the importance of chit1, Kp infected chit1$^{-/-}$ mice exhibited a significant protected phenotype where they lost significantly lower weight compared to the wild type mice, suggesting better health in these mice (Fig. 3A). In agreement to the overall health, the chit1$^{-/-}$ mice have significantly lower bacterial dissemination to the spleen (Fig. 3B), a marker of bacterial dissemination, while maintaining similar bacterial load in the lung as well as similar protein levels in the BAL (a marker of lung leakage) (Fig. 3C and D). In agreement with similar bacterial burden and airway protein content, no difference in pathology was observed between the two groups after infection (Fig. 4 K & L). However, the dissemination phenotype was visible at early time points as indicated by decreased bacterial counts in the blood of chit1$^{-/-}$ mice (Fig 5 A). Also, the trend toward decreased bacterial number in the blood of chit1$^{-/-}$ mice suggest that the significant decreased bacterial load in the spleens of chit1$^{-/-}$ mice is due to limited dissemination from the lung rather than better peripheral control of infection. This theory is further supported by the fact that similar dissemination and health was observed in chit1$^{-/-}$ mice when infected by intra-peritoneal route (Sup Fig. 3). Chitotriosidase is believed to be important mediator of host response against pathogens based on its presence in macrophages where it performs important functions during pulmonary infections. It is interesting to note that it was hypothesized that chit1 might possess antibacterial properties by itself or it can promote antibacterial activity of lysozymes (40). Experiments showed that chit1 does not possess any bactericidal activity of its own or boost the bactericidal activity of lysozymes (40). Our study shows a better control of bacterial infection in chit1$^{-/-}$ mice, which further refutes any direct antibacterial role of chitotriosidase against Kp *in vivo.* In this regard, our data suggest that not only chitotriosidase is dispensable for immunity against Kp but plays a detriments role in Kp lung infection. Further, these effects are specific to Kp lung infection. In a mouse model of Pseudomonas lung infection, we did not observe any protection in chit1−/− mice, indeed out data show that they had higher bacterial load in the lung and spleen. Although Pseudomonas and Klebsiella are both gram-negative pathogens associated with nosocomial lung infections, they have many differences in their virulence factors and patho-mechanisms. Flagella and type-3 secretion system constitute important virulence factor in PA infection, while capsule is important component of Kp virulence (41, 42). Their virulence also varies to great extent in our mouse models, where inoculation of only 5×10^3 is sufficient to cause a severe lung infection which effectively disseminates to other organs for Klebsiella. On the other hand, 1×10^7 CFUs of PA are needed to establish severe infection in the lungs, with minimal dissemination to the periphery. These obvious differences might account for differences observed in Chit1−/− mice between these two pathogens in this study.

The controlled dissemination in chit1$^{-/-}$ mice during Kp infection resulted in increased survival, further emphasizing the advantage provided by chit1 deficiency during Kp

infection. However, the survival advantage provided by chit1 deficiency was limited to just one day extension of survival in our infection model (median survival 3 day vs 4 day, Fig. 5 B). This might be attributed to the high lethality of Kp and its ability to keep multiplying in the mouse in spite of an intact immune system. We also used antibiotics in our model to mimic a clinical scenario; patients with pneumonia often treated with antibiotics (43). Two doses of the antibiotic treatment increased survival in both the groups compared to the mice that did not receive antibiotics (Fig 5B vs 5D). However, chit1$^{-/-}$ mice had significantly increased survival upon antibiotic treatment compared to the wild type mice that received similar antibiotic treatment (Fig. 5D).

To understand the mechanisms that improved bacterial control in these mice, we sought to determine the inflammatory response in these mice, as inflammation plays an important role in limiting bacterial growth and spread during Kp lung infection (44, 45). Lung infection produced a similar inflammatory response in wild type and chit1$^{-/-}$ mice at 48 h, as indicated by the number of recruited cells including neutrophils and macrophages, which are the major cell types in the airways of Kp infected mice (Fig 4 A, B & C). We also characterized inflammation at early time points post infection and found a similar inflammatory response, even at early phase of infection (data not shown), which suggests that chit1 has a limited impact on the inflammatory response during Kp bacterial infection. In agreement with these results, chit1$^{-/-}$ mice invoked similar levels of inflammatory responses upon administration of LPS in the lung (Sup Fig 4 A, B & C).

Cytokines contribute to the orchestration of the inflammatory response (45–47). Levels of many cytokines, both inflammatory and anti-inflammatory, were measured to find out if chit1 plays a role in regulating cytokine response during lung infection. After infection, both wild type and chit1$^{-/-}$ mice produced similar levels of cytokines in their lungs (Fig. 4 D-J), suggesting a limited role of chit1 in regulating cytokine response during Kp lung infection. These data explain similar number of cell recruitment in the lung during infection in chit1$^{-/-}$ mice. In agreement with these *in vivo* data, bone marrow derived macrophages from wild type and chit1$^{-/-}$ mice, or macrophage cell line RAW 264.7 upon treatment with chitotriosidase, produced similar cytokines levels (Sup Fig. 4). These data exclude the role of chit1 in regulating the inflammatory response to Kp bacteria or bacterial product LPS.

To elucidate the mechanisms that might contribute to the improved outcome in lung infection, we explored the regulation of the Akt pathway during lung infection. True chitinase AMCase, and chitinase-like protein BRP 39 have been shown to regulate Akt activity (30, 48). The Akt pathway plays an important role in both immune and structural cells. It has been reported that Akt activation can increase the phagocytic activity in macrophages to engulf more of bacteria while also increases extracellular trap formation in neutrophils (49, 50). Similarly, in epithelial cells, Akt has been shown to control bacterial transmigration across the gut epithelial monolayer (51). In this study, we observed that, while at baseline, chit1$^{-/-}$ mice maintained a lower level of Akt activation, but upon infection with Kp, chit1$^{-/-}$ had a robust increase in Akt activation (Fig. 6 A & B). Similar to the whole lung Akt level, bone marrow derived macrophages from chit1$^{-/-}$ mice had a lower Akt phosphorylation at baseline. However, upon stimulation with LPS, chit1$^{-/-}$ bone marrow derived macrophages had robust Akt activation which was absent in wild type

macrophages (Fig. 6 C). This altered activation of Akt in chit1−/− macrophages was associated with increased ability to control growth of Kp *in-vitro*. We believe that a contribution of both immune cells and structural cells might have contributed to the altered dissemination phenotype we observed in chit1−/− during Kp infection. We also determined the role of chit1 in regulating the MAPK pathway by measuring the activation of P42/44 and P38 MAPK proteins. There was no significant difference in both the MAPK protein levels either at baseline or upon infection (Fig. 6 D-I).

To understand the role of Akt, we used a pharmacological inhibitor of Akt in our Kp infection mouse model. Akt inhibition led to a significant impairment of the host's ability to maintain health and survival by impairing bacterial clearance and increasing bacterial dissemination (Fig. 7), suggesting the importance of the Akt pathway in Kp lung infection.

Various theories have been proposed to explain the presence of chitinase in mammals. Earlier evidence suggested increased chitinase deficiency among the Caucasian population while a conserved presence was observed in Africans (21). This study indicated that improved living conditions in Caucasians have led to enzyme deficiency while the continuous threat of malaria and other parasitic infections provided selection pressure to retain intact chit1 gene in Africans (21). The same group demonstrated elevated levels of chitotriosidase in the colostrum of African women compared to Caucasian women (52). However, later observations did not support these initial reports. Studies demonstrated no significant correlation between parasitic information and chit1 genotype (33, 53, 54). Furthermore, high prevalence of chitinase deficiency was observed in Peruvians with high prevalence of enteroparasites and high consumption of chitin containing food, refuting previous beliefs that chitinases play a role in the digestion of chitin containing food or protection against parasitic infection (33). The obvious role of chit1 in fungal infection was proposed due to its *in vitro* fungicidal activity (15, 25), but this role was refuted by a recent study using an *in vivo* fungal infection model (55). In this study, chit1 deficiency was shown to provide a survival advantage in a Cryptococcal infection model by limiting pathological inflammation mediated by chitotriosidase mediated chitin recognition (55). This study refutes that chit1 has an important role in the host defense against fungal infections.

It is interesting to compare the effects of chit1 in lung infection with other members of chitinase and chitinase-like proteins. Our lab has reported that chitinase 3 like protein1 (Chil1) plays an essential role in lung infection with *Streptococcus*. Chil1 protects macrophages against *Streptococcus* induced pyroptosis, which leads to improved bacterial clearance and limited pathology (56). Similarly, in *Pseudomonas* lung infection, absence of chil1 results in an exaggerated inflammatory response and a diminished survival (57), suggesting its essential role in both gram positive and gram-negative bacterial infections. These studies show distinct roles between different chitinases and chitinase-like proteins.

Overall, our study suggests that chitinase activity is actively regulated during bacterial lung infection, mainly by proteolytic cleavage of chitotriosidase mediated by infiltrated neutrophils. Deficiency of chit1 provides a survival advantage to the host during lung infection by limiting bacterial dissemination. This might provide a new therapeutic target to increase host immunity during bacterial infections. These data also suggest that the loss of

chitinase activity in humans provides an edge during bacterial lung infection, putting a selection pressure for mutant gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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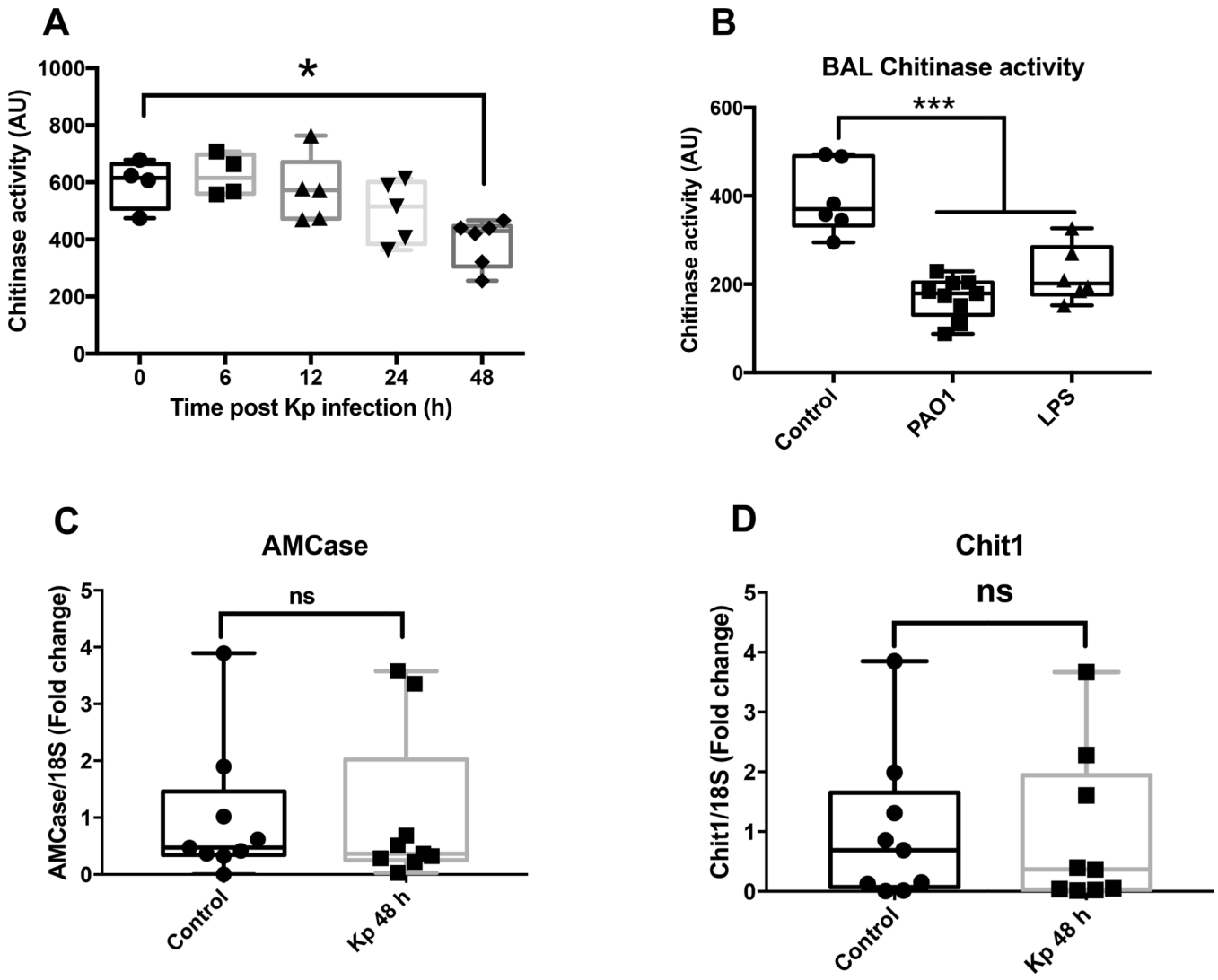

Fig. 1. Down-regulation of chitinase activity during infection and inflammation.

Chitinase activity was measured in the BAL samples of C57BL6 mice infected with 5×10^3 CFUs of Kp for indicated time points (A) or in mice administered with LPS or Pseudomonas aeruginosa strain PAO1 for 12 h (B). Lung expression of true chitinases and AMCase (C) and Chit1 (D) were measured using qpcr method. *, $P \leq 0.05$, **, $P \leq 0.01$, ns = not significant, AU = arbitrary unit. Data are from at one of at least two independent experiments performed for A and B, N = 3–5 each, while data are pooled from two independent experiments in C and D, N =4–5 each experiment.

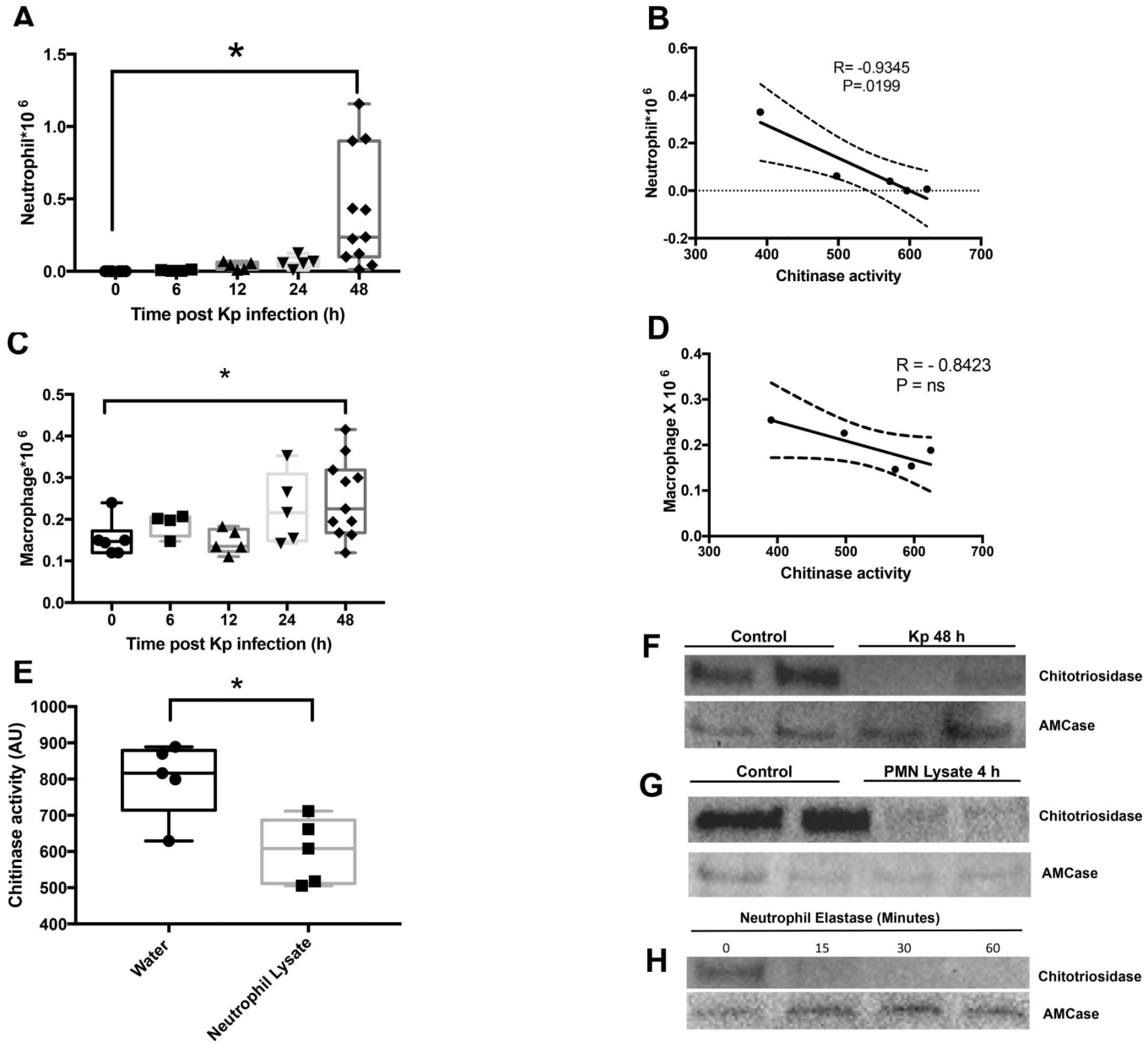

Fig. 2. Neutrophils mediate decrease in chitinase activity in mice.

Time course of neutrophil infiltration during Kp lung infection (A) and its correlation with chitinase activity (B). Time course of macrophage recruitment to the lung during Kp infection (C) and its correlation with chitinase activity (D). Mice were administered with neutrophil lysate directly to the lung and BAL samples were collected after 4 h of incubation to measure chitinase activity (E). Chit1 (upper gel) and AMCase levels (lower gels) in the BAL samples of Kp infected mice (F). Chit1 (upper gel) and AMCase levels (lower gels) in the BAL samples of neutrophil lysate administered mice (G). Mice BAL samples were treated with neutrophil elastase for indicated time points and probed for chit1 (upper gel) and AMCase (lower gel) (H). *, $P \leq 0.05$, ns = not significant, AU = arbitrary unit. Data are pooled from one or two independent experiments performed at each time point (A, C), N

=4–5 each experiment or from one representative experiment from at least two independently performed experiments (B, D and E - I), N = 4–5 each group.

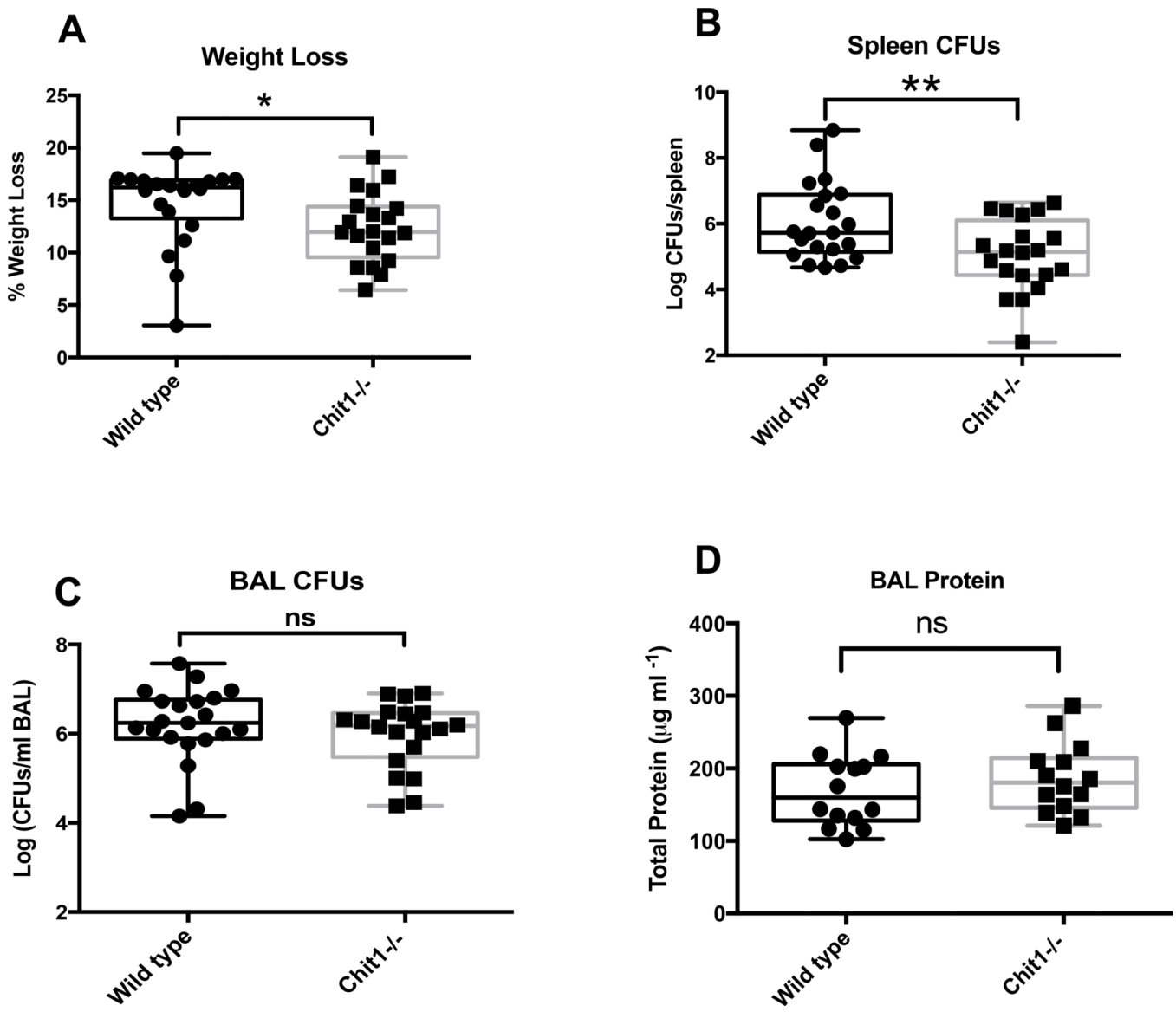

Fig. 3. Chit1 regulates overall health and bacterial dissemination during Kp lung infection.

Wild type and chit1$^{-/-}$ mice were infected with Kp for 48 h to measure their weight loss (A), bacterial burden in the spleen (B), and bacterial burden in the BAL (C) and total protein content in the BAL (D). *, P≤ 0.05, **, P≤ 0.01 using unpaired t test, ns = not significant. Data are pooled from 4 independent experiments (N = 4–6 each experiment).

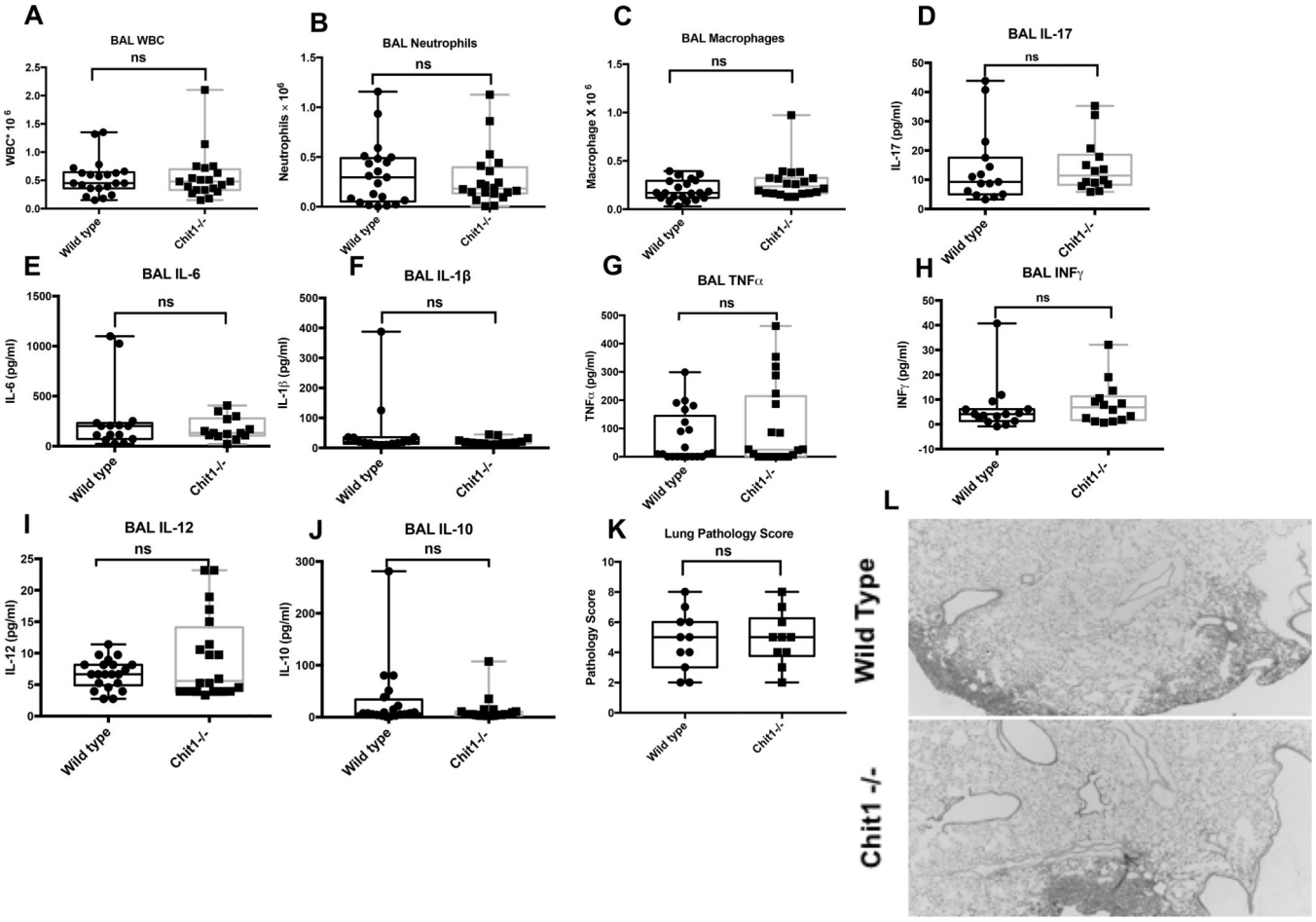

Fig. 4. Chit1$^{-/-}$ mice have similar level of inflammation and lung pathology during Kp lung infection.

Total WBCs were counted in the BAL samples of WT and Chit1$^{-/-}$ mice at 48 h post infection (A). Neutrophil (B) and Macrophage (C) numbers were assessed in the BAL samples. BAL samples from wild type and chit1$^{-/-}$ mice infected with 5 X 10 ^{3} CFUs for 48 h were used to measure cytokine levels using either Bioplex cytokine assay kit (TNFα, IL-10 and IL-12) or sandwich ELISA (IL-6, IL-1β, INFγ and IL-17) (D-J). Lung sections were stained with H & E and scored for pathology based on peribronchial and perivascular inflammation. Representative lung section from two groups (L), and the lung pathology scores (K), ns = not significant. Data are pooled from 4 independent experiments performed (A –J, N = 4–6 each experiment) or from two independent experiments (K & L).

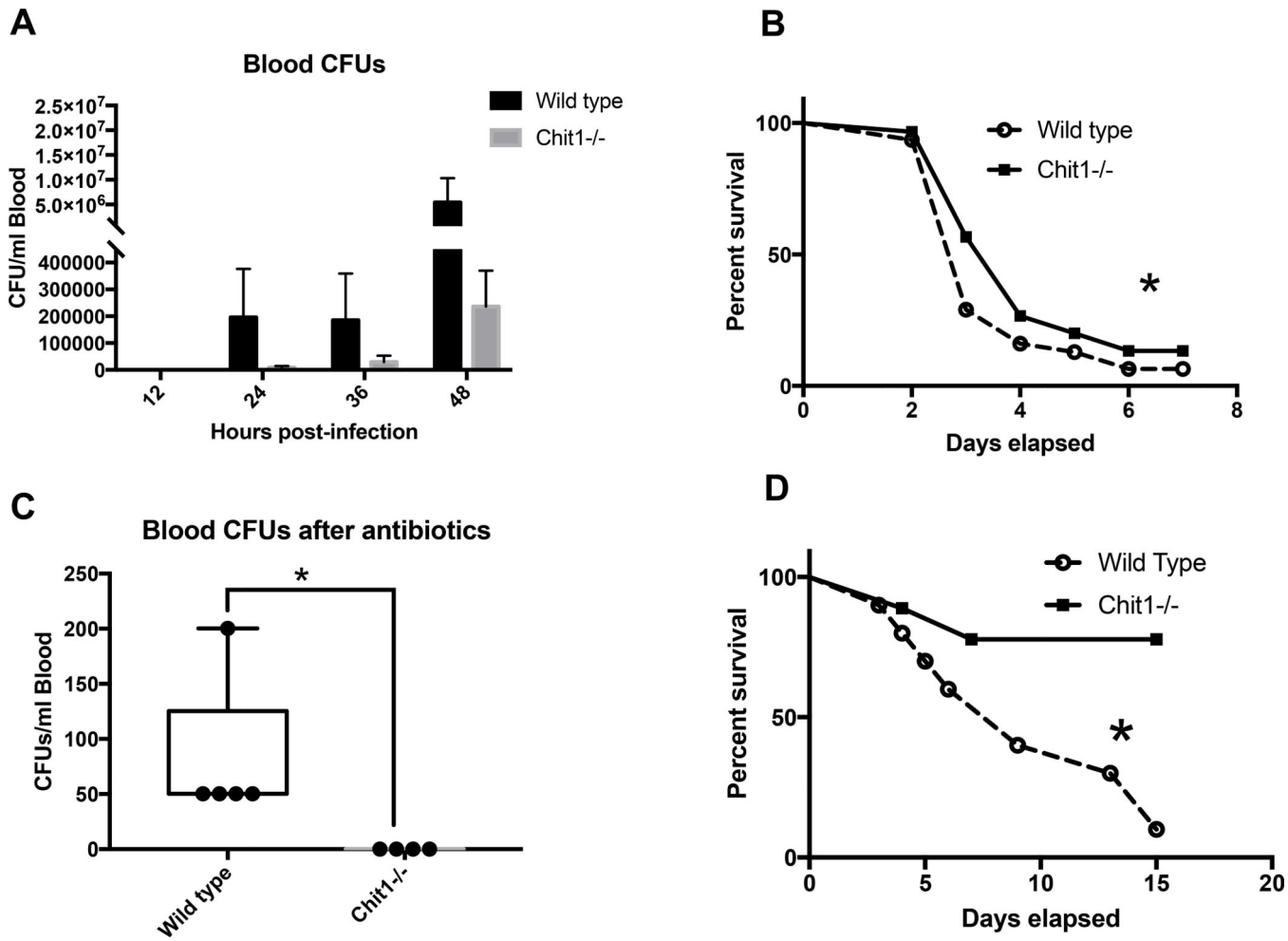

Fig. 5. Chit1$^{-/-}$ mice have early control on bacterial dissemination and improved survival during Kp lung infection.

Wild type and chit1$^{-/-}$ mice were infected with 5X10^3 CFUs of Kp and blood samples were collected at given time points to determine bacterial numbers (A). Wild type and chit1$^{-/-}$ mice were infected with 5X10^3 CFUs of Kp and monitored for survival for 7 days (B). Wild type and chit1$^{-/-}$ mice were infected with 5X10^3 CFUs of Kp and treated with two doses of gentamicin at 48 and 60 h post infection. Blood was harvested at 72 h post infection to determine bacterial burden in the blood (C). Mice were observed for survival after two doses of antibiotics (D). *, P≤ 0.05 using Wilcoxon test. N = 10–12 each group for A, 30–31 each group for B, 4–5 for C and 9–10 each group for D. Data are pooled from two independent experiments (A and D, N = 4–5 each experiment) or one of two independent experiment with same outcome (C, N =4 −5) or pooled from 4 independent survival studies (B, N =5–6 each experiment).

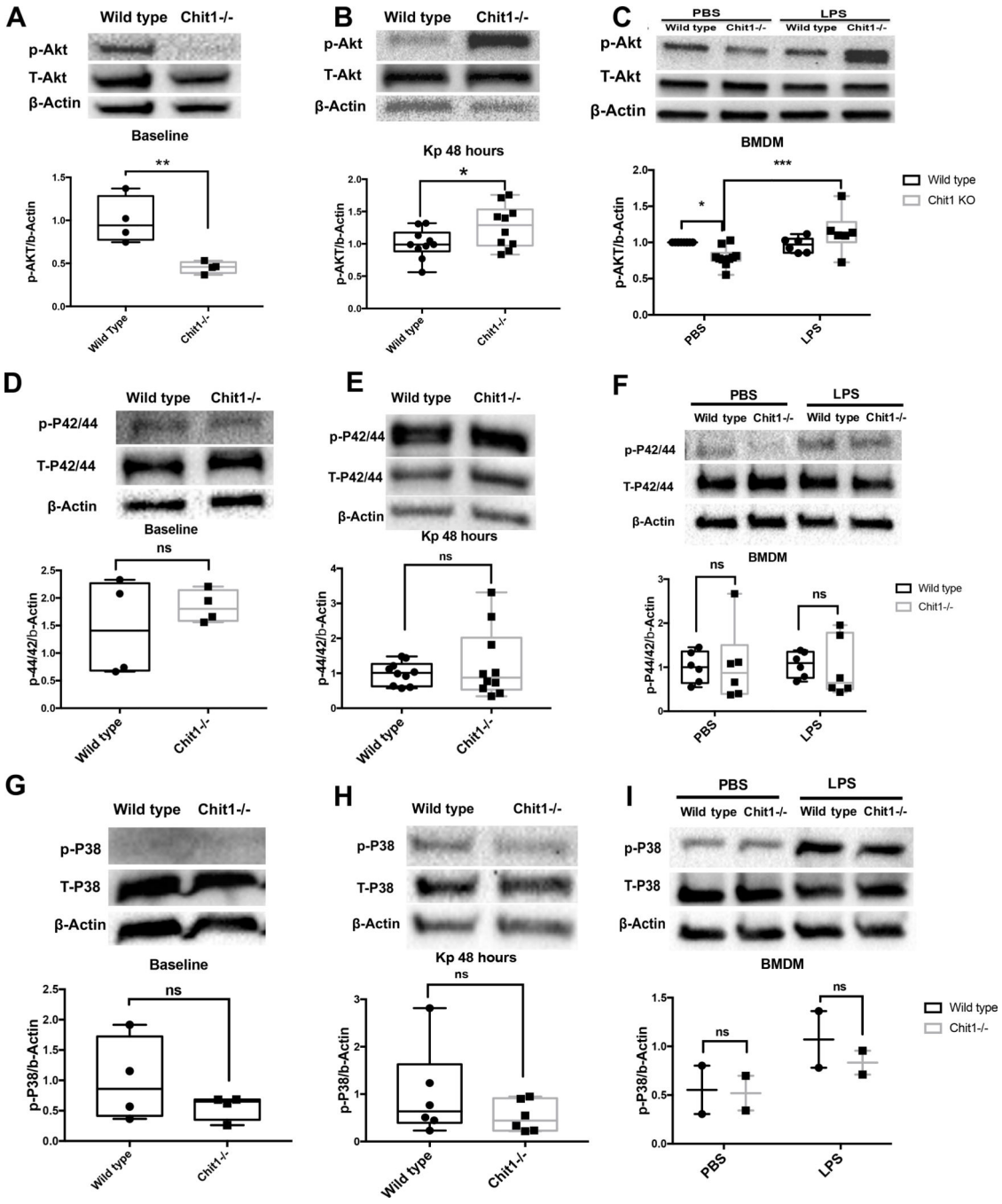

Fig. 6. Chit1$^{-/-}$ mice have altered Akt activation during Kp lung infection.

Lung lysates from wild type and chit1$^{-/-}$ mice either at baseline (A) or after 48 h of Kp infection were tested for Akt activation (B). Akt activation was also tested in BMDMs from wild type and chit1$^{-/-}$ mice stimulated with PBS or LPS for two h (C). Levels of phosphorylated MAPK P42/44 and P38 at baseline (D & G), lung infection (E & H) and in BMDMs (F & I) were measured and normalized to β actin. Upper panels are representative images while the lower panels are the densitometry quantifications of the bands. *, P≤ 0.05, **, P≤ 0.01, using t test. Each experiment was performed at least two independent

experiments (A & B, N =4–6 each experiment). All the in-vitro experiments were performed at least 3 times in duplicates (C-I).

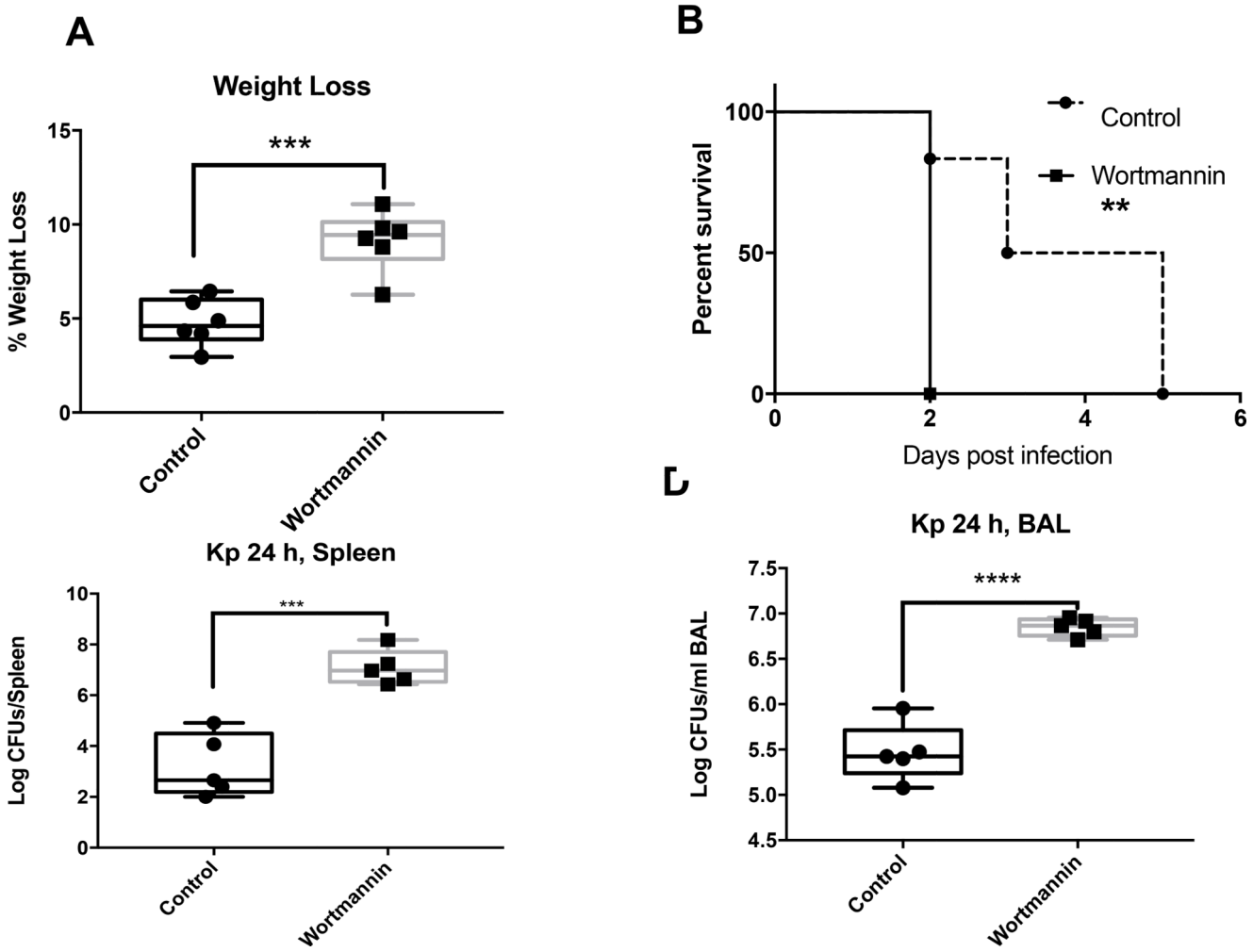

Fig. 7. Akt inhibition impairs bacterial clearance and survival in Kp lung infection

Mice were infected with Kp and injected with Akt pathway inhibitor wortmannin at 1 mg/Kg. Mice were measured for weight loss (A) and observed for survival (B). Bacterial load in BAL and spleen were measured (C & D). *, P≤ 0.05, **, P≤ 0.01, P≤ 0.005 using unpaired t test or Sidak's multiple comparisons test or Wilcoxon test for survival. Data are from one experiment each performed independently for bacterial load and survival study (N= 5–6 each experiment).