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Protective role of Mincle in bacterial pneumonia by regulation of neutrophil mediated phagocytosis and extracellular trap formation

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Abstract

Background: Nosocomial infections with *Klebsiella pneumoniae* (KPn) are a frequent cause of Gram-negative sepsis. To understand the functioning of host innate immune components in this disorder, we examined a previously uninvestigated role of C-type lectin receptor Mincle, in pneumonic sepsis caused by KPn.

Methods: Disease progression in wild-type and Mincle$^{-/-}$ mice undergoing pulmonary infection with KPn was compared.

Results: While the wild-type mice infected with a sublethal dose of bacteria could resolve the infection with bacterial clearance and regulated host response, the Mincle$^{-/-}$ mice were highly susceptible with a progressive increase in bacterial burden despite their ability to mount an inflammatory response which turned to an exaggerated hyperinflammation with the onset of severe pneumonia. This correlated with severe lung pathology with a massive accumulation of neutrophils in their lungs. Importantly, Mincle$^{-/-}$ neutrophils displayed a defective ability to phagocytize non-opsonic bacteria and an impaired ability to form extracellular traps (NETs), an important neutrophil function against invading pathogens, including KPn.

Conclusion: Our results demonstrate protective role of Mincle in host defense against KPn pneumonia by coordinating bacterial clearance mechanisms of neutrophils. A novel role for Mincle in regulation of NETosis may have implications in chronic disease conditions characterized by deregulated NET formation.

Introduction

Lower respiratory tract infection with bacteria can lead to sepsis development, which is a complex immune disorder characterized by a systemic hyperinflammation. There are currently no effective therapies for sepsis that results in 750,000 hospitalizations annually in the United States with a mortality rate of 20-50% [1, 2]. Nosocomial infections with opportunistic pathogen *Klebsiella pneumoniae* (KPn) account for 5-20% of Gram-negative sepsis cases [3-5]. Additionally, emergence of multidrug resistant isolates of KPn in clinical settings is a serious health concern. As innate mucosal immunity plays a direct role in bacterial killing and immunomodulation in this acute infection [6-10], an understanding of functioning of host innate immune components might provide targets for modulation of host immune system in a beneficial manner.

Mincle is a C-type lectin receptor (CLR) belonging to the Dectin-2 subfamily of innate immune receptors that can function as an activating receptor for host- as well as pathogen-associated molecular patterns, termed alarmins and PAMPs respectively (reviewed in [11, 12]. It is an inducible receptor, expressed mainly by myeloid cells such as macrophages, neutrophils, myeloid dendritic cells as well as some B-cell subsets [11, 13, 14]. Functional analysis of this receptor in macrophages has received the most attention, where its association with FcRγ activates downstream signaling cascades involving Syk kinases resulting in induction of protective inflammatory response [15-17]. While the function of Mincle in chronic bacterial infections such as tuberculosis and fungal infections was examined in these studies, its role in acute pneumonic infections leading to sepsis development has not been explored. Furthermore its

functions other than as an inflammatory PRR (**P**attern **R**ecognition **R**eceptor) have received little, if any, attention.

Neutrophil mediated responses are essential for combating pneumonic bacterial infection and their protective role in sepsis and KPn infection in particular has been described [18, 19]. The professional antimicrobial program of neutrophils mainly constitutes phagocytosis of infectious agents followed by production of noxious agents such as reactive oxygen species which kill the internalized microbes. Another recently established mechanism of microbial killing by neutrophils is by formation of extracellular traps (termed neutrophil extracellular traps or NETs) which are DNA fibrils expelled by these cells that are decorated with granular contents such as various proteases and can ensnare and kill the microbes without phagocytosis [20-22]. Mincle has been shown to be expressed by neutrophils and while shown to play a role in neutrophil mediated protective responses against Candida and mycobacteria [23, 24], its direct role in bacterial phagocytosis and NET formation is not known. Since neutrophils are a key cell type in controlling KPn infection, Mincle signaling in neutrophils may be a key event in control of KPn infection and sepsis.

In this study we examined the role of Mincle in acute KPn infection causing pneumonic sepsis. Our results suggest novel protective function of Mincle as non-opsonic phagocytic receptor for the bacteria and in regulation of neutrophil NET formation indicating the importance of this CLR in neutrophil-specific bacterial clearance mechanisms in pneumonic infections.

Methods

Bacterial strains and Mice

The KPn (ATCC strain 43826) were grown to log phase in LB medium at 37°C. All *in-vivo* experiments were performed using 6-8 weeks old female wild-type C57BL/6 or Mincle$^{-/-}$ mice on same background obtained from the Consortium of Functional Genomics, Scripps, La Jolla and bred in the animal facility of the University of North Dakota. The animals were used according to institutional and federal guidelines.

Infection of Mice, survival and bacterial burden

Mice were anaesthetized with a mixture of 30mg/ml ketamine and 4 mg/ml xylazine in PBS and were infected intranasally with sublethal dose (2.5 x 10^4 bacteria in 20ul of saline, determined experimentally) of KPn or with 20 µl of saline alone. Survival of the mice was recorded for up to 2 weeks post-infection (p.i.). In some experiments, the mice were euthanized at indicated times p.i. and blood, lungs and liver were aseptically homogenized in cold PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, serially diluted homogenates and blood were plated on LB agar and incubated at 37°C overnight.

Quantitative real-time PCR

Total RNA from lungs of infected and mock control mice harvested at various times p.i. was extracted using Trizol reagent (Invitrogen) according to the manufacturers' instructions. Real-time PCR analysis was performed using SYBR green (Applied Biosystems, CA, USA) to measure the expression levels of Mincle-specific mRNA by using specific primers (sense) 5'-ACC AAA TCG CCT GCA TCC -3' and (anti-sense) 5'- CAC TTG GGA GTT TTT GAA GCA

TC -3' (as described by us in [25]. The target gene expression levels were normalized to levels of the house keeping 18S gene in the same sample. Expression of Mincle in infected samples was determined as fold change over that in control samples as calculated by using the formula $2^{-(\Delta\Delta Ct)}$.

Multi-analyte profile analysis

The lung homogenates were prepared as described for the bacterial burden analysis above and were centrifuged at 2000 x g for 15 min to clear cellular debris. The supernatants were immediately frozen at -80^{o} C. The biomarker levels in lung homogenates were determined commercially by Myriad Rules-based Medicine (Austin, TX, USA) utilizing a multiplexed analysis.

Histological analysis

For histological analysis, frozen lung tissues were processed as previously described [26, 27]. Serial horizontal sections (10 μm thick) of frozen lung tissues thus obtained were stained with hematoxylin and eosin for pathological analysis as previously described [28, 29] .

Flow Cytometry

Lungs or BAL cells were harvested from mice at 3 days p.i. and processed as previously described by us [26, 27, 30]. Enumeration of neutrophils by flow cytometry (using a BD LSR II, Becton Dickinson, San Jose, CA) was done by quantitating Ly6G+CD11b+ cells stained with Pacific Blue^{TM} anti-mouse CD11b and APC anti-mouse Ly6G (Clone 1A8) antibodies (Biolegend, San Diego, CA). Mincle expression was examined by using a rat anti-mouse Mincle

monoclonal antibody (Clone 6G5, InvivoGen, CA) followed by goat anti-rat Alexa-488 secondary antibody (InvitroGen, OR). FlowJo (Tree Star) software was used to analyze all data.

Bacterial phagocytosis by neutrophils

Bacterial phagocytosis of WT and Mincle$^{-/-}$ neutrophils was assessed by flow cytometry. For this peritoneal neutrophils were isolated using an established model of thioglycollate-induced peritonitis. Sterile 4% thioglycollate was injected in peritoneal cavity of mice and neutrophils enriched 8-12h following the injection were isolated (95-99% pure as assessed by flow cytometry using GR1 and CD11b antibodies). Neutrophils were incubated with GFP-labeled KPn (kindly provided by Dr. Steven Clegg, University of Iowa) for 1 hour and washed three times with ice-cold FACS-buffer (PBS+10% fetal bovine serum). Fluorescence of the attached but non-internalized bacteria was quenched by treating the cells with 0.04% Trypan Blue. The % positive cells containing fluorescent bacteria were determined by flow-cytometry using uninfected neutrophils as control.

Neutrophil NETs

For detection of neutrophil NETs *in-vivo*, the bronchoalveolar lavage (BAL) was performed in WT and Mincle$^{-/-}$ mice at 3dp.i.. The lavage cells were cytocentrifuged on glass slides and were co-stained with Sytox Green (Molecular Probe, Eugene, OR) and rabbit anti-neutrophil elastase (NE) polyclonal antibody (abcam,) followed by goat anti-rabbit Alexa546 antibody. The percent NET formation was quantitated by dividing the number of NET-forming neutrophils by total number of cells in 8-10 random microscopic fields and multiplying the values by 100.

Statistical Analysis

Statistical analysis of survival studies was performed by Kaplan Meir log-rank test; bacterial burdens by non-parametric Mann-Whitney Test. All other statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA).

RESULTS

Mincle is highly expressed in lungs during pneumonic KPn infection.

To examine the role of innate immune receptors in pathogenesis of KPn induced pneumonic sepsis, we initially screened a panel of 52 membrane-bound and soluble CLRs by Taqman Low-density arrays which showed an upregulated expression of Dectin-2 family CLR, Clec4e (also called Mincle), among others in the lungs of mice undergoing respiratory KPn infection. To further confirm the Mincle expression, real-time quantitative PCR was performed using RNA from lungs of KPn infected wild-type mice. The results showed a progressive increase in the transcript level of Mincle mRNA which was maximally transcribed by 3dp.i. and remained at high level throughout the course of infection (Figure 1*A*). Flow cytometry analysis further confirmed the increased numbers of Mincle-positive cells, majority of which were CD11b+Ly6G+ neutrophils, in the lungs of KPN-infected mice (Figure 1*B*). This indicated that Mincle was highly expressed on neutrophils and played a role in pathogenesis of KPn pneumonia.

Mincle deficient mice are highly susceptible to KPn pneumonia.

To examine the role of Mincle in disease development, overall disease severity and survival was compared in wild-type and Mincle$^{-/-}$ mice infected with a sub-lethal dose of KPn. This dose was

experimentally determined at which the WT mice displayed minimal morbidity and mortality [29]. As shown in Figure 1*C*, 76% of WT mice infected with $2.5x10^4$ CFUs of KPn survived the infection with transient signs of disease (ruffled fur, lethargy) early during infection and appeared healthy later. The Mincle$^{-/-}$ mice, in contrast, were extremely susceptible to this dose and all mice succumbed to infection by day 6p.i. While majority of the infected WT mice cleared the infection by day 5 p.i., Mincle$^{-/-}$ mice exhibited progressive development of disease and overt signs of infection (weight loss, piloerection, hunched gait, lethargy, increased respiratory rate). The increased susceptibility of Mincle-/- mice clearly indicated a protective role played by this CLR during pneumonic KPn infection.

Mincle deficiency results in increased bacterial burden and systemic dissemination

In order to examine if increased susceptibility of Mincle$^{-/-}$ mice to KPn infection correlated with inefficiency to clear bacteria, homogenized lungs, liver and blood from infected Mincle$^{-/-}$ and WT mice collected at various times post infection were plated on LB agar. Up to 2dp.i., Mincle$^{-/-}$ and WT animals displayed similar bacterial burdens in their lungs (Figure 2*A*). By 3dp.i., however, lungs of Mincle$^{-/-}$ mice exhibited significantly higher bacterial counts as compared to their WT counterparts. The bacterial burden in these mice remained high at 5dp.i., the time when majority of mice had become moribund. In contrast, the WT mice displayed 3-5 logs lower bacterial burden at 3dp.i. and the counts continued to drop through 5dp.i., indicating clearance of bacteria and resolution of the infection in these mice. The Mincle$^{-/-}$ mice also displayed a higher systemic dissemination of bacteria as depicted by significantly higher bacterial load in liver (Figure 2*B*) and a more severe bacteremia (Figure 2*C*). In contrast, no viable bacteria were

detected in the blood of WT mice by 5dp.i. These data indicated that Mincle mediated responses directly or indirectly influenced bacterial clearance in pneumonic infection with KPn.

Mincle$^{-/-}$ mice exhibit hyperinflammatory response

We next examined if the inability of Mincle$^{-/-}$ mice to clear the bacteria was due to a defect in mounting inflammatory response. In both WT and Mincle$^{-/-}$ strains, mock infected mouse lungs displayed similar low basal levels of inflammatory cytokines tested (Figure 3). Upon KPn infection, WT mice exhibited increased levels of these cytokines at 1dp.i., which started to drop by 3dp.i. and were reduced to minimal levels by 5dp.i. (Figure 3). This was consistent with the reduced bacterial burden in these mice at these times p.i.. In contrast, infection of Mincle$^{-/-}$ mice resulted in a progressive increase in levels of these cytokines through the course of infection, which remained high till the mice became moribund. These mice in fact exhibited an overwhelming inflammatory response at 3d and 5dp.i. (Figure 3). The levels of inflammatory cytokines and chemokines tested in lungs of these mice were significantly higher than WT mice at these time points. The levels of IL-10, an anti-inflammatory cytokine were also significantly higher in Mincle$^{-/-}$ mice, suggesting a condition of "cytokine storm" typical of sepsis, where anti-inflammatory host mediators are upregulated in an attempt to counter-balance the systemic inflammatory response [31-33]. These results show that Mincle deficiency did not render the mice defective in their ability to mount an inflammatory response but these mice rather displayed a hyperinflammatory phenotype typically associated with sepsis. Our observations thus raised the possibility that Mincle likely plays a direct role in bacterial clearance and the hyperinflammation resulted due to activation of other PRRs and inflammatory receptors in response to persistent overwhelming bacterial burden in Mincle-/- mice undergoing pneumonic KPn infection.

Effect of Mincle deficiency on neutrophil infiltration and overall lung pathology

Since neutrophils are a key cell type involved in bacterial clearance and initiation of protective immune response during KPn pneumonia, we next compared neutrophil infiltration and gross immunopathological changes in KPn infected WT and Mincle$^{-/-}$ mice. The mock control mice of both strains displayed similar normal lung tissue morphology in H&E stained sections (Figure 4). A moderate transient infiltration of immune cells was observed in infected WT mice by day 3p.i. which was reduced substantially by 5dp.i. The overall architecture of the lungs was largely preserved in the WT animals throughout the infection. The Mincle$^{-/-}$ mice, on the other hand, displayed a progressive increase in immune cell infiltration, which were mainly neutrophils, based on characteristic multi-lobed nuclei (Figure 4 inset). By day 3 p.i. substantially increased influx of cells was observed in large lesions and by 5 p.i., extensive foci of consolidation were visible with massive accumulation of neutrophils around alveolar spaces (Figure 4). Flow cytometry analysis of infiltrating cells in lungs confirmed that the majority of these cells were Ly6G+CD11b+ neutrophils (Figure 5*A*). The numbers of these cells were significantly higher in the infected Mincle$^{-/-}$ lungs than those in the WT mice (Fig. 5*A*, bar graph). This correlated with significantly higher levels of neutrophil chemoattractants (CXCL1, CXCL6), neutrophil survival mediator (GM-CSF) and neutrophil activation markers (MMP9, MPO) in these mice, as compared to their WT counterparts (Figure 5*B*).

Mincle$^{-/-}$ neutrophils are defective in KPn phagocytosis

We next examined the bacterial uptake by Mincle$^{-/-}$ neutrophils, in light of an increased bacterial burden in Mincle$^{-/-}$ mice. For this, phagocytosis of GFP-labeled KPn was compared between WT and Mincle$^{-/-}$ neutrophils by flow cytometry. As shown in Figure 6, Mincle deficiency resulted in

significantly reduced phagocytosis of non-opsonized bacteria by neutrophils. The uptake of opsonized bacteria was also reduced in Mincle$^{-/-}$ neutrophils as compared to the Mincle-sufficient WT cells, however the differences were not statistically significant. These results indicate that Mincle is likely a novel non-opsonic phagocytic receptor for KPn and plays an important role in bacterial uptake by neutrophils.

Mincle$^{-/-}$ neutrophils are defective in NET formation

Extracellular trap formation is an important mechanism by which neutrophils clear extracellular bacteria. Since Neutrophil NET mediated killing has been shown to play a role in KPn clearance [6, 34], we saught to determine if Mincle deficiency resulted in a defect in NET formation. In order to minimize the tissue processing to avoid degradation of NETs, neutrophils isolated from BAL were used. Flow cytometry analysis showed that neutrophils were a predominant cell-type in the BAL of infected WT and Mincle-/- mice (Figure 7*A*). A quantitative comparison of BAL neutrophils showed that significantly higher numbers Mincle sufficient WT neutrophils produced NETs (Figure 7*B*) which stained positive for neutrophil-specific enzyme neutrophil elastase (Figure 7*C*), showing that these fibrillar structures originated mainly from neutrophils. Furthermore, the NETs observed in Mincle$^{-/-}$ neutrophils appeared dwarfed and lacked the web-like appearance as observed in the WT mice. This observation, together with reduced phagocytic ability of Mincle$^{-/-}$ neutrophils shows that Mincle deficiency severely impairs neutrophil mediated bacterial uptake and clearance mechanisms in lungs during pneumonic KPn infection.

DISCUSSION

Pneumonic sepsis is a major health care burden worldwide and *K. pneumoniae* (KPn) is the most frequent Gram-negative sepsis-associated opportunistic pathogen [2]. An imbalance of innate

immune responses resulting in deleterious and prolonged inflammation and impairment of protective functions of first responder cells such as neutrophils have been directly correlated with sepsis-associated mortality [35-37]. This warrants an improved understanding of functioning of innate immune components in this deadly disease. In this study, we sought to determine the role of Mincle, an innate immune C-type lectin receptor in KPn pneumonia. Here we report several novel findings: 1) We show, for the first time, a clear phenotype in terms of a severely reduced survival rate of Mincle$^{-/-}$ mice upon pneumonic KPn infection; 2) the reduced survival is not due to a defect in ability to mount an inflammatory response in absence of Mincle; 3) Mincle acts as a non-opsonic phagocytic receptor mediating uptake of KPn by neutrophils; and 4) Mincle deficiency results in a defect in neutrophil NET formation upon KPn infection. Our results thus show that Mincle is required for defense against KPn induced pneumonic sepsis and that lack of Mincle causes a defect in neutrophil mediated bacterial clearance mechanisms such as phagocytosis and NET formation.

Mincle has been previously shown to play a role in eliciting inflammatory responses against Mycobacterium, *Candida albicans* and skin fungal pathogens, Malassezia and Fonsecaea [17, 38-40]. In these infections, Mincle expressed on macrophages, upon recognition of its ligands triggers FcRγ-Syk-Card9 pathway to induce production of protective Th1/Th17 responses as well as chemokines required for recruitment of inflammatory cell types [16, 39, 41]. The increased susceptibility to these infections in the absence of Mincle was measured in terms of increased bacterial and fungal burden which was attributed to reduced inflammatory response and defective pathogen clearance in these studies. However, overall survival of the experimental animals was not affected by Mincle deficiency. Our study for the first time, reports a clear outcome where Mincle seems to play a non-redundant role in survival of KPn infected

pneumonic mice. Moreover, the reduced survival of Mincle$^{-/-}$ mice is not due to their inability to mount an inflammatory response. These mice, instead exhibit hyperinflammation in their lungs suggesting that the protective ability of Mincle was independent of its role in eliciting inflammatory response. It is likely that the redundant function of other PRRs, upon recognition of bacterial PAMPs and endogenous alarmins generated from increased bacterial growth and accumulation of dead cells over time, is sufficient to induce inflammation in the absence of Mincle. Indeed, Mincle-/- mice in our studies, exhibited overwhelming local as well as systemic bacterial burdens.

Concomitant to increased bacterial burden, Mincle$^{-/-}$ mice exhibited extensive neutrophil accumulation, the primary cell type shown to play an important role in mediating protective immune response against Kpn infection [4, 7, 18, 42]. We thus examined if Mincle$^{-/-}$ neutrophils were defective in performing cellular functions such as internalization of bacteria via phagocytosis which would explain increased bacterial burden in Mincle$^{-/-}$ mice despite a heightened inflammation. Indeed, Mincle$^{-/-}$ neutrophils showed a mitigated phagocytosis of non-opsonized, but not opsonized KPn, suggesting a non-redundant and direct role of Mincle for internalization of non-opsonized bacteria. To the best of our knowledge, this is the first study reporting Mincle as a non-opsonic phagocytic receptor. Ongoing studies in our lab are currently investigating Mincle specific ligand of KPn, nature of this interaction and production of specific antibodies that can inhibit this interaction. Lectinophagocytosis or lectin-mediated uptake by macrophages has been reported previously for several pathogens (reviewed in [43]. However, the receptors or mechanisms of non-opsonized phagocytosis of bacteria by neutrophils are poorly understood. Non-opsonic phagocytosis by receptors like Mincle may be important during early stages of infection before the onset of humoral immunity to generate opsonins, and in

complement deficient or immunosuppressed patients. This mode of phagocytosis is particularly significant for inhaled bacteria as serum and complement components are as such limited in the alveolar space [44]. Although appearance of serum components in alveolar space is common during severe KPn pneumonia, owing to high binding capacity of Mincle to mannose and N-acetyleglucosamine [45], uptake by Mincle of KPn with mannan-rich capsule in lungs could be a major mechanism of bacterial clearance in lungs. Absence of Mincle and a resulting defect in initial phagocytic uptake of KPn, as observed in our studies likely contributes to the increased bacterial burden and subsequent inflammation via activation of other PRRs such as TLRs.

One of the more recently defined mechanisms of antimicrobial activity of neutrophils is extrusion of fibrous mesh of chromatin that entraps extracellular pathogens [20]. These structures, termed NETs, are decorated with antimicrobial factors normally contained within neutrophil granules and represent an important strategy of neutrophils to immobilize and kill pathogens. Our observation reported in this study that Mincle$^{-/-}$ neutrophils are defective in NET formation *in-vivo* during KPn infection coincides with overwhelming bacterial burdens in these mice. These results are in line with previous reports indicating that NET mediated killing is an important mechanism of bacterial clearance and protection against KPn induced pneumonia [6, 34]. How Mincle regulates NET formation is currently under investigation in our laboratory and is expected to provide novel insights into mechanism of NETosis. This will have important implications in chronic disease conditions where deregulated NET formation is associated with the pathophysiology.

Taken together, our results show that Mincle plays a protective role in KPn induced pneumonic sepsis by regulating neutrophil phagocytosis and NET formation, two important mechanisms of

antimicrobial activity of neutrophils. Particularly, the novel observation of Mincle as a potential new component of NETosis pathway implicates this CLR in a much wider range of biological functions that initially surmised.

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Footnotes

Conflict of Interest

The authors have no financial conflict of interest.

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Figure Legends

Figure 1. Mincle is highly induced in the lungs of KPn infected pneumonic mice and Mincle deficiency increases susceptibility to the infection. A. Total RNA was extracted by Trizol method from the lungs of KPn infected wild-type C57/BL6 mice, harvested at indicated time post-infection. The mRNA levels of Mincle were analyzed by real-time PCR as described in Methods and are expressed as fold changes over the levels in mock control mice calculated by using the formula $2^{-(\Delta\Delta Ct)}$. Data shown are the averages of 6-8 mice per group in two independent experiments. **B.** Mincle expression was examined on infiltrating lung cells from KPn infected wild-type C57/BL6 mice harvested at 3dp.i. by flow cytometry using a rat anti-mouse Mincle antibody followed by goat anti-rat secondary antibody labeled with Alexa-488. Mincle positive cells were gated and further analyzed for expression of CD11b and Ly6G as mentioned in the Methods section. The dot plots shown are representative of 3 independent experiments with 3 mice each. **C.** Fifteen each WT and Mincle$^{-/-}$ mice were intranasally infected with $2.5X10^4$ CFUs of *Klebsiella pneumonia* (KPn) in 20µl of sterile PBS and were assessed daily for disease severity. The survival was monitored for two week. Statistical comparison of susceptibility was done by Kaplan-Meier survival curve statistical analysis ($p<0.001$).

Figure 2. Mincle$^{-/-}$ mice display increased bacterial burden and systemic dissemination during pneumonic KPn infection. WT and Mincle$^{-/-}$ were intranasally infected with $2.5X10^4$ CFUs of KPn. At indicated times post infection the mice were sacrificed, systemic organs were isolated, homogenized and plated as described in Materials and Methods. Bacterial burden was enumerated after incubating the plates overnight at 37^{o}C. The data shown is from three independent experiments with 3-5 mice at each time point per experiment. Significant

differences in bacterial burden (using non-parametric Mann-Whitney test) in WT and Mincle$^{-/-}$ are denoted by asterisks (*, p<0.05; **, p<0.005, ***p<0.001).

Figure 3. Pneumonic Mincle$^{-/-}$ mice exhibit hyperinflammatory response. The lungs from mock control and KPn infected WT and Mincle$^{-/-}$ mice were harvested at indicated time points post-infection, homogenized in PBS with protease inhibitors and analyzed commercially for host immune mediators by rodent multi-analyte profile (Myriad^{TM} Rules-Based Medicine, Austin, TX). Results shown are average of 3-4 each infected and mock control mice from 3 independent experiments. Amounts of mediators shown were significantly higher (***p<0.001) in KPn infected Mincle$^{-/-}$ mice at 3dp.i. and 5dp.i. in comparison with their levels in the infected WT mice at those time points tested.

Figure 4. Pneumonic Mincle$^{-/-}$ mice exhibit severe lung pathology characterized by massive neutrophil accumulation. Hematoxylin & Eosin staining of lung cryosections from mock control and KPn infected WT and Mincle$^{-/-}$ mice isolated at indicated times post-infection. Magnification 100X. Inset shows a highly magnified area (1000X) of a lesion in infected Mincle$^{-/-}$ lung depicting neutrophils as indicated by characteristic multilobed nuclear morphology.

Figure 5. Increased neutrophil accumulation coincides with elevated expression of neutrophil chemoattractant and activation markers in lungs of KPn infected Mincle$^{-/-}$ mice. **A.** Flow cytometry analysis of Ly6G+CD11b+ neutrophils in mock control (WT-M and Mincle$^{-/-}$-M) and KPn infected (WT-Inf and Mincle$^{-/-}$-Inf) WT and Mincle$^{-/-}$ mice. Total lungs cells were isolated from mice by collagenase treatment at 3dp.i.. The cells were stained with anti-Ly6G-

APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. The bar graph shows average of total number of neutrophils in lungs of 2-3 mock control and 3-4 KPn infected WT and Mincle$^{-/-}$ mice each from 3 independent experiments. Dot plots shown on the right are from one representative experiment. Statistical significance are denoted by asterisks (***, $p<0.001$). **B.** The lungs from mock control and KPn infected WT and Mincle$^{-/-}$ mice were harvested at indicated time points post-infection and analyzed commercially for host immune mediators by rodent multi-analyte profile (Myriad^{TM} Rules-Based Medicine, Austin, TX). Levels of neutrophil chemoattractants (CXCL2, CXCL6, GM-CSF) and activation markers (matrix metalloproteinase 9, MMP-9 and myeloperoxidase, MPO) shown are average of 3-4 each infected and mock control mice from 3 independent experiments. Amounts of mediators shown were significantly higher (***$p<0.001$) in Mincle$^{-/-}$ mice at 3dp.i. and 5dp.i. in comparison with their levels in the WT mice at those time points tested.

Figure 6. Mincle deficiency impairs neutrophil phagocytosis of non-opsonized bacteria. Peritoneal neutrophils from WT and Mincle$^{-/-}$ mice were incubated with GFP (Green Fluorescent Protein)-labeled KPn with (opsonized) or without (non-opsonized) 10% normal mouse serum for 1 hour followed by quantitation of phagocytosis by flow cytometry. The results are expressed as % cells positive for fluorescent bacteria. Significant differences are denoted by asterisks (**, $p<0.005$).

Figure 7. Mincle deficiency causes a defect in formation of neutrophil extracellular traps (NETs) in the lungs of mice upon pneumonic KPn infection. A. Enumeration of CD11b+Ly6G+ neutrophils by flow cytometry in BAL isolated from KPn infected WT and Mincle$^{-/-}$ mice. **B.** Quantitation of neutrophils showing NETs in BAL isolated from WT and

Mincle$^{-/-}$ mice infected with KPn. (***p<0.001). **C.** Representative fluorescence images of the neutrophils isolated from BAL fluid of WT (upper panel) and Mincle$^{-/-}$ (lower panel) mice infected with KPn, and stained with Sytox Green to label DNA (green) and a rabbit anti-neutrophil elastase (NE) polyclonal antibody followed by goat anti-rabbit Alexa546 (red). The neutrophils from WT mice showed web-like structures that stained positive for NE and Sytox green (white arrow), while the Mincle-/- neutrophils appeared inactive and displayed occasional small DNA fibers that lacked the typical web-like appearance of NETs (blue arrow). The experiment was repeated 3 times with 3-4 mice in each group. Magnification 400X.

Figure 1

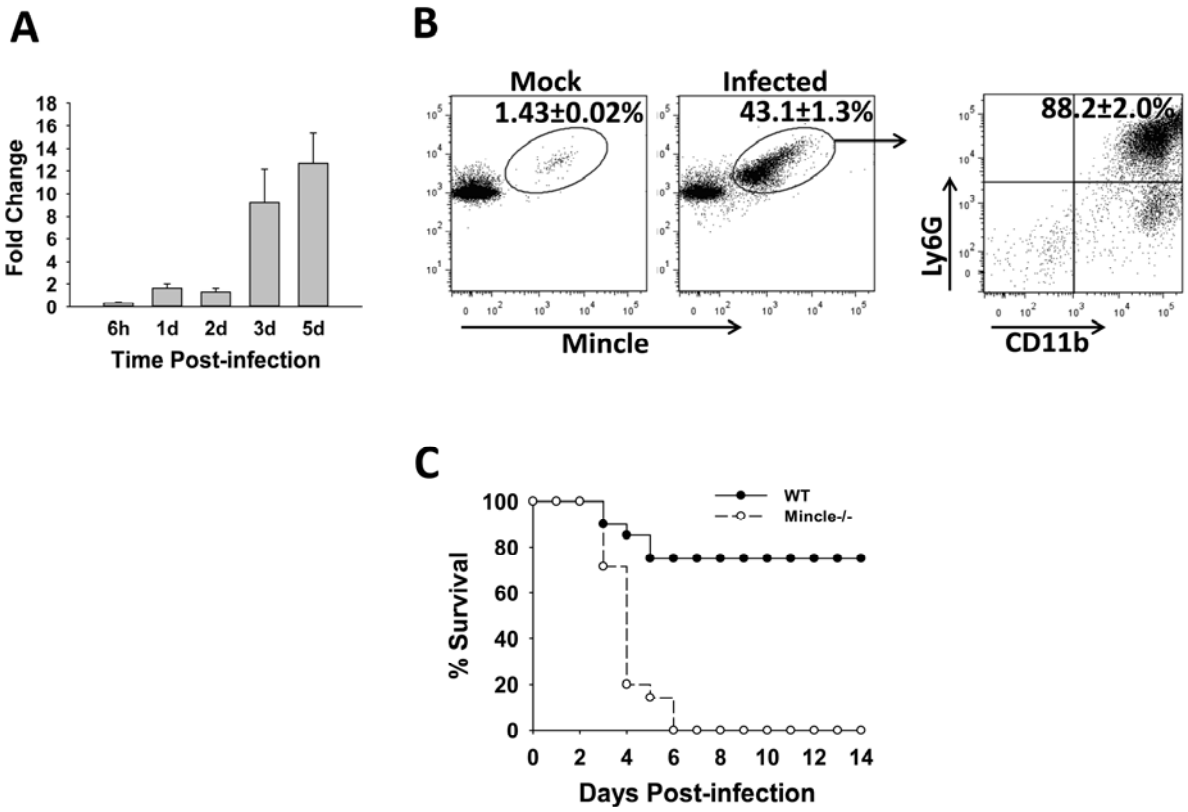

Figure 2

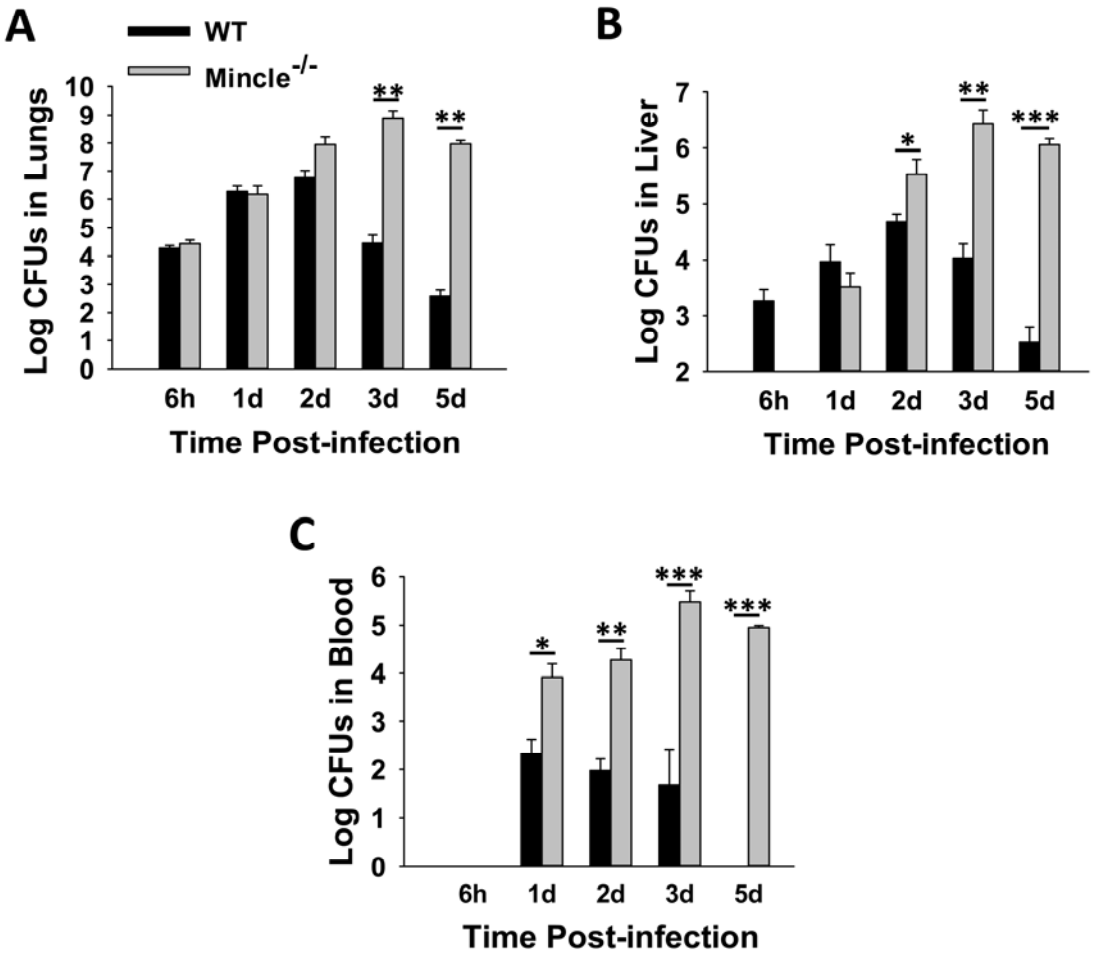

Figure 3

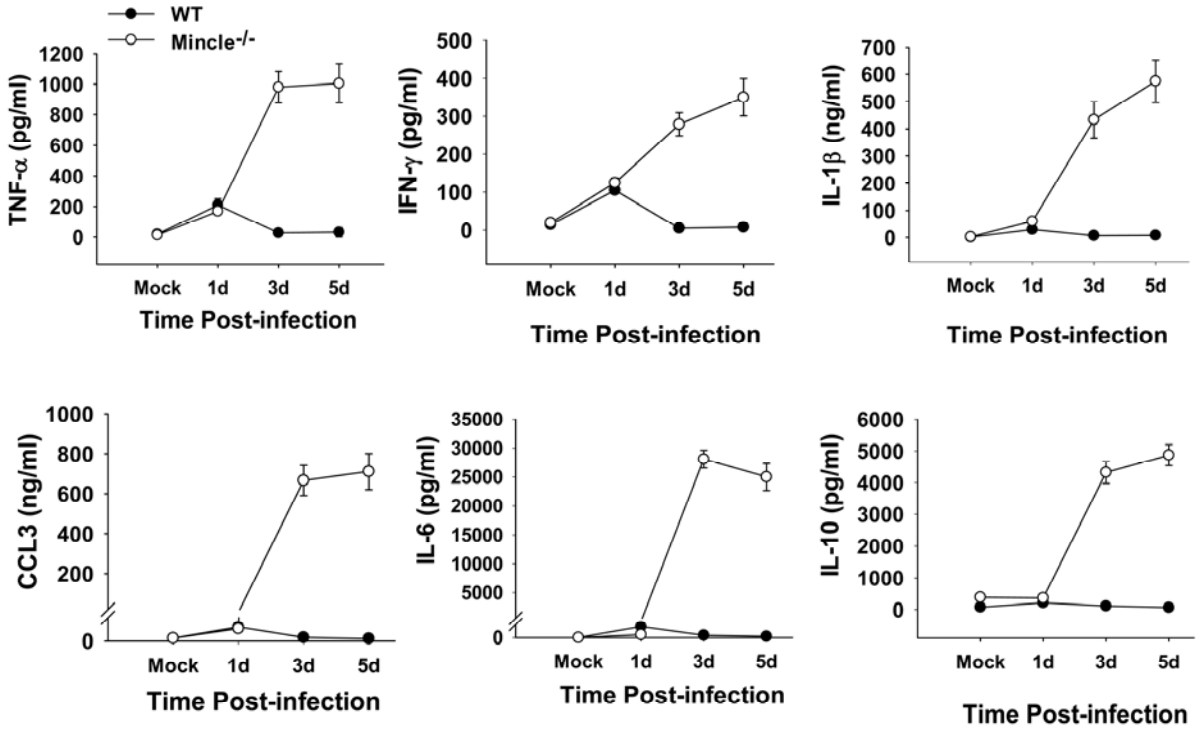

Figure 4

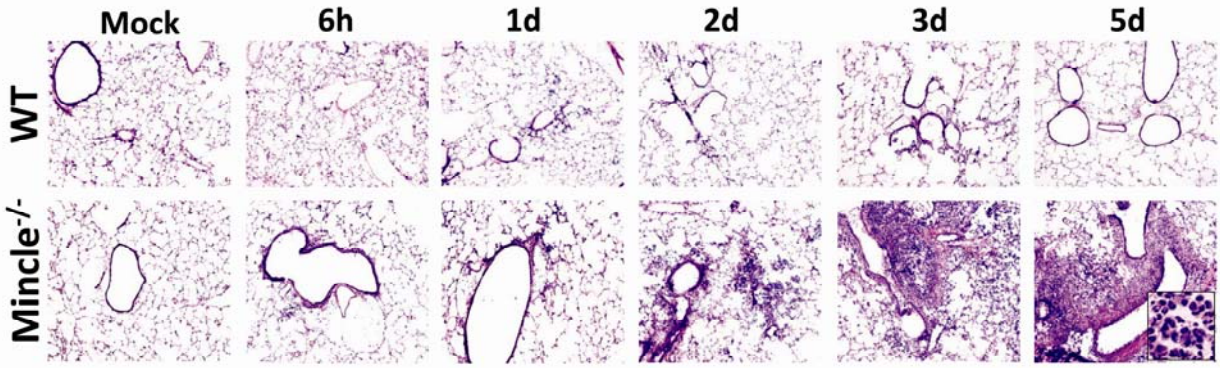

Figure 5

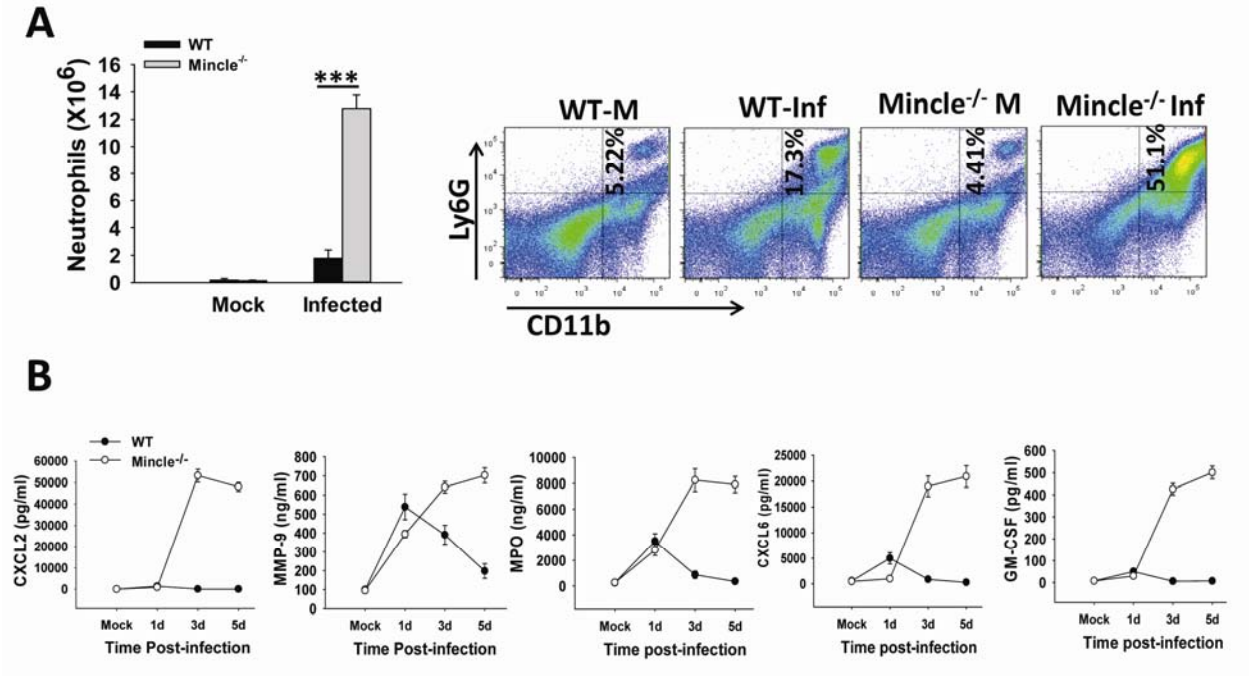

Figure 6

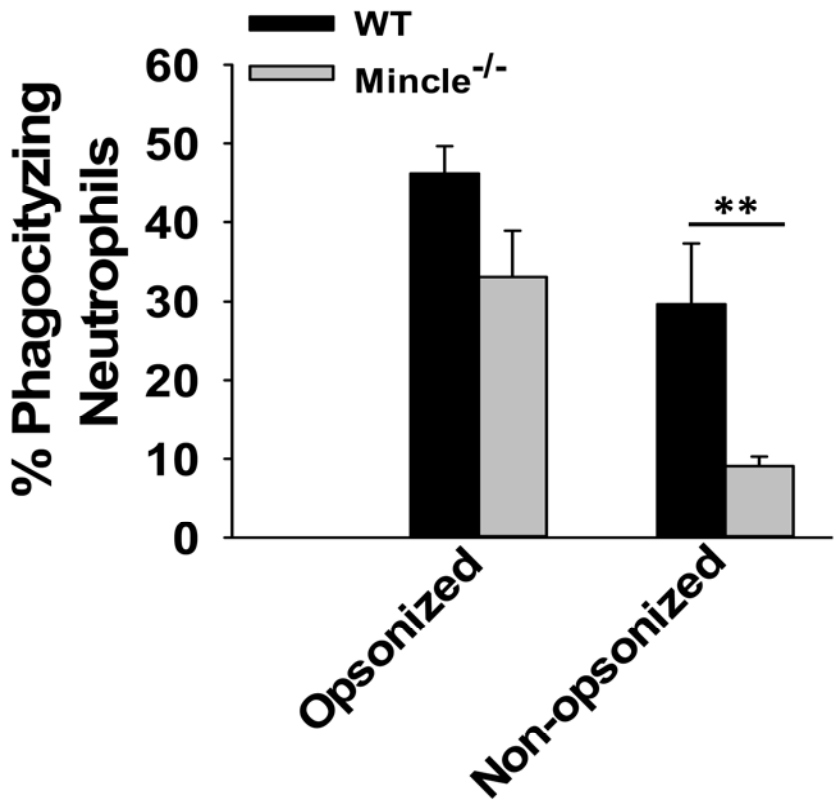

Figure 7