Protective Role of Mincle in Bacterial Pneumonia by Regulation of Neutrophil Mediated Phagocytosis and Extracellular Trap Formation

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

**Methods.** Disease progression in wild-type and Mincle−/− mice undergoing pulmonary infection with *K. pneumoniae* was compared.

**Results.** Whereas 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 that 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 phagocytose nonopsonic bacteria and an impaired ability to form extracellular traps (NETs), an important neutrophil function against invading pathogens, including *K. pneumoniae*.

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

**Keywords.** Mincle; Clec4e; Klebsiella; pneumonia; sepsis; neutrophils; NETs; phagocytosis.

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, which results in 750,000 hospitalizations annually in the United States and has a mortality rate of 20%–50% [1, 2]. Nosocomial infections with the opportunistic pathogen *Klebsiella pneumoniae* account for 5%–20% of cases of gram-negative bacterial sepsis [3–5]. Additionally, emergence of multidrug-resistant isolates of *K. pneumoniae* in clinical settings is a serious health concern. Because 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 the 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-associated molecular patterns (alarmins) and pathogen-associated molecular patterns (PAMPs) [11, 12]. It is an inducible receptor, expressed mainly by myeloid cells such as macrophages, neutrophils, myeloid dendritic cells, and some B-cell subsets [11, 13, 14]. Functional analysis of this receptor in macrophages has received the most attention, where its association with FcRy 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 pneumonia was examined in these studies, its role in acute pneumonia infections leading to sepsis development has not been explored. Furthermore, its functions other than as an inflammatory pattern-recognition receptor (PRR) have received little, if any, attention.

Neutrophil-mediated responses are essential for combating pneumonia bacterial infection, and their protective role in sepsis and \textit{K. pneumoniae} 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, that kill the internalized microbes. Another recently established mechanism of microbial killing by neutrophils is by their formation of extracellular traps (NETs), which are DNA fibrils expelled by neutrophils and are decorated with granular contents, such as various proteases, that can ensnare and kill microbes without phagocytosis [20–22]. Mincle has been shown to be expressed by neutrophils, and although it plays a role in neutrophil-mediated protective responses against \textit{Candida} species and mycobacteria [23, 24], its direct role in bacterial phagocytosis and NET formation is not known. Because neutrophils are a key cell type in controlling \textit{K. pneumoniae} infection, Mincle signaling in neutrophils may be a key event in control of \textit{K. pneumoniae} infection and sepsis.

In this study, we examined the role of Mincle in acute \textit{K. pneumoniae} infection causing pneumonia sepsis. Our results suggest a novel protective function of Mincle as a nonopsonic phagocytic receptor for bacteria and in regulation of NET formation, indicating the importance of this CLR in neutrophil-specific bacterial clearance mechanisms in pneumonic infections.

\section*{METHODS}

\subsection*{Bacterial Strains and Mice}

\textit{K. pneumoniae} (ATCC strain 43 826) was grown to log phase in LB medium at 37°C. All in vivo experiments were performed using 6–8-week-old female wild-type (WT) C57BL/6 or Mincle \textsuperscript{−/−} mice on the same genetic background (Consortium of Functional Genomics, Scripps, La Jolla, CA) that were bred in the animal facility of the University of North Dakota. The animals were used according to institutional and federal guidelines.

\subsection*{Infection Survival, and Bacterial Burden in Mice}

Mice were anesthetized with a mixture of 30 mg/mL ketamine and 4 mg/mL xylazine in phosphate-buffered saline and were infected intranasally with a sublethal dose (2.5 \times 10^7 bacteria in 20 μL of saline, determined experimentally) of \textit{K. pneumoniae} or with 20 μL of saline alone. Survival of the mice was recorded for up to 2 weeks after infection. In some experiments, the mice were euthanized at indicated times after infection, and blood, lungs, and liver were aseptically homogenized in cold phosphate-buffered saline with Complete \textsuperscript{+} Protease Inhibitor Cocktail (Roche Diagnostics Basel, Switzerland). For the bacterial burden analyses, serially diluted homogenates and blood were plated on LB agar and incubated at 37°C overnight.

\subsection*{Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis}

Total RNA from lungs of infected and mock control mice harvested at various times after infection was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers’ instructions. Real-time PCR analysis was performed using SYBR green (Applied Biosystems, Carlsbad, CA) to measure the expression levels of Mincle-specific messenger RNA by using the following specific primers, as described by us elsewhere [25]: sense, 5′-ACC AAA TCG CCT GCA TCC-3′; and antisense, 5′-CAC TTG GGA GTT TTT GAA GCA TC-3′. 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 the fold change over that in control samples, calculated as 2^(-ΔΔCt).

\subsection*{Multianalyte Profile Analysis}

The lung homogenates were prepared as described above for the bacterial burden analysis and were centrifuged at 2000 \times g for 15 minutes to clear cellular debris. The supernatants were immediately frozen at −80°C. The biomarker levels in lung homogenates were determined commercially by Myriad RBM (Austin, TX), using a multiplexed analysis.

\subsection*{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].

\subsection*{Flow Cytometry}

Lungs or bronchoalveolar lavage cells were harvested from mice 3 days after infection and were 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\textsuperscript{+}CD11b\textsuperscript{+} cells stained with hematoxylin and eosin for pathological analysis, as previously described [28, 29].

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\subsection*{Bacterial Phagocytosis by Neutrophils}

Bacterial phagocytosis of WT and Mincle \textsuperscript{−/−} neutrophils was assessed by flow cytometry. Peritoneal neutrophils were isolated using an established model of thioglycollate-induced peritonitis. Sterile 4% thioglycollate was injected in the peritoneal
cavity of mice, and neutrophils enriched for 8–12 hours following the injection were isolated (95%–99% pure as assessed by flow cytometry using GR1 and CD11b antibodies). Neutrophils were incubated with green fluorescent protein (GFP)–labeled K. pneumoniae (kindly provided by Dr Steven Clegg, University of Iowa) for 1 hour and washed 3 times with ice-cold fluorescence-activated cell sorter buffer (phosphate-buffered saline plus 10% fetal bovine serum). Fluorescence of the attached but noninternalized bacteria was quenched by treating the cells with 0.04% trypan blue. The percentage of neutrophils containing fluorescent bacteria, was determined by flow cytometry, using uninfected neutrophils as a control.

NETs
Detection of NETs in vivo, bronchoalveolar lavage was performed in WT and Mincle−/− mice 3 days after infection. The lavage cells were cytocentrifuged on glass slides and were stained with Sytox Green (Molecular Probe, Eugene, OR) and rabbit anti-neutrophil elastase polyclonal antibody (Abcam), followed by goat anti-rabbit Alexa546 antibody. The percentage of neutrophils forming NETs was quantitated by dividing the number of NET-forming neutrophils by the total number of neutrophils 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 were evaluated by the nonparametric Mann–Whitney U 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 K. pneumoniae Infection
To examine the role of innate immune receptors in the pathogenesis of K. pneumoniae–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 Mincle, among other CLRs, in the lungs of mice undergoing respiratory K. pneumoniae infection. To further confirm Mincle expression, real-time quantitative PCR was performed using RNA from lungs of K. pneumoniae–infected WT mice. The results showed a progressive increase in the transcript level of Mincle messenger RNA, the transcription of which was maximally transcribed by 3 days after infection and remained at a high level throughout the course of infection (Figure 1A). Flow cytometry further confirmed the increased numbers of Mincle-positive cells, the majority of which were CD11b+Ly6G+ neutrophils, in the lungs of K. pneumoniae-infected mice (Figure 1B). This indicated that Mincle was highly expressed on neutrophils and played a role in the pathogenesis of K. pneumoniae pneumonia.

Mincle−/− Mice Are Highly Susceptible to pneumonic K. pneumoniae infection
To examine the role of Mincle in disease development, overall disease severity and survival were compared in WT and Mincle−/− mice infected with a sublethal dose of K. pneumoniae. This dose was experimentally determined to be one at which the WT mice displayed minimal morbidity and mortality [29]. As shown in Figure 1C, 76% of WT mice infected with 2.5 × 10^4 colony-forming units of K. pneumoniae survived the infection, with transient signs of disease (eg, ruffled fur and lethargy) early during infection and a healthy appearance later. The Mincle−/− mice, in contrast, were extremely susceptible to this dose, and all mice succumbed to infection by day 6 after infection. Whereas majority of infected WT mice cleared the infection by day 5, Mincle−/− mice exhibited progressive development of disease and overt signs of infection (eg, weight loss, piloerection, hunched gait, lethargy, and increased respiratory rate). The increased susceptibility of Mincle−/− mice clearly indicated a protective role played by this CLR during pneumonic K. pneumoniae infection.

Mincle Deficiency Results in Increased Bacterial Burden and Systemic Dissemination
To examine whether the increased susceptibility of Mincle−/− mice to K. pneumoniae infection correlated with an inefficient clearance of bacteria, homogenized lungs, liver, and blood from infected Mincle−/− and WT mice, collected at various times after infection, were plated on LB agar. Up to 2 days after infection, Mincle−/− and WT animals displayed similar bacterial burdens in their lungs (Figure 2A). By 3 days after infection, however, lungs of Mincle−/− mice exhibited significantly higher bacterial counts, compared with their WT counterparts. The bacterial burden in these mice remained high at day 5 after infection, the time when the majority of mice had become moribund. In contrast, the WT mice displayed a 3–5-log lower bacterial burden 3 days after infection, and the counts continued to decrease through day 5 after infection, indicating the clearance of bacteria and the resolution of the infection in these mice. The Mincle−/− mice also displayed a higher systemic dissemination of bacteria, as depicted by a significantly higher bacterial load in the liver (Figure 2B) and more-severe bacteremia (Figure 2C). In contrast, no viable bacteria were detected in the blood of WT mice by day 5 after infection. These data indicated that Mincle-mediated responses directly or indirectly influenced bacterial clearance in pneumonic infection with K. pneumoniae.

Mincle−/− Mice Exhibit Hyperinflammatory Response
We next examined whether the inability of Mincle−/− mice to clear bacteria was due to a defect in mounting an inflammatory response. In both WT and Mincle−/− strains, mock-infected
mouse lungs displayed similar low basal levels of inflammatory cytokines tested (Figure 3). Upon *K. pneumoniae* infection, WT mice exhibited increased levels of these cytokines on day 1 after infection, which started to decrease by 3 days after infection and were reduced to minimal levels by 5 days after infection (Figure 3). This was consistent with the reduced bacterial burden in these mice at these times after infection. In contrast, infection of Mincle−/− mice resulted in a progressive increase in levels of these cytokines through the course of infection, which remained high until the mice became moribund. These mice exhibited an overwhelming inflammatory response 3 and 5 days after infection (Figure 3). The levels of inflammatory cytokines and chemokines tested in lungs of these mice were significantly higher than those in WT mice at these time points. The levels of interleukin 10, an anti-inflammatory cytokine, were also significantly higher in Mincle−/− mice, suggesting a cytokine storm typical of sepsis, in which anti-inflammatory host mediators are upregulated in an attempt to counterbalance 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 that, instead, these mice 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 that the hyperinflammation was attributable to activation of other PRRs and inflammatory receptors in response to a persistent overwhelming bacterial burden in Mincle−/− mice undergoing pneumonic *K. pneumoniae* infection.

**Effect of Mincle Deficiency on Neutrophil Infiltration and Overall Lung Pathology**

Because neutrophils are a key cell type involved in bacterial clearance and initiation of the protective immune response during *K. pneumoniae* pneumonia, we next compared neutrophil infiltration and gross immunopathological changes in *K. pneumoniae*-infected WT and Mincle−/− mice. The mock control mice of both strains displayed similar normal lung tissue morphology in hematoxylin and eosin–stained sections (Figure 4). A moderate transient infiltration of immune cells

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**Figure 1.** Mincle is highly induced in the lungs of *Klebsiella pneumoniae* infected pneumonic mice, and Mincle deficiency increases susceptibility to the infection. A, Total RNA was extracted by the Trizol method from the lungs of *K. pneumoniae*-infected wild-type (WT) C57/BL6 mice, harvested at the indicated times after infection. The messenger RNA levels of Mincle were analyzed by real-time polymerase chain reaction as described in Methods and are expressed as fold changes over the levels in mock control mice, calculated as $2^{-\Delta\Delta Ct}$. Data are the averages of 6–8 mice per group in 2 independent experiments. B, Mincle expression was examined by flow cytometry on infiltrating lung cells harvested from *K. pneumoniae*-infected WT C57/BL6 mice. Cells were harvested 3 days after infection and stained, 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 Methods. The dot plots are representative of 3 independent experiments with 3 mice each. C. Fifteen each of WT and Mincle−/− mice were intranasally infected with 2.5 × 10⁴ colony-forming units of *K. pneumonia* in 20 μL of sterile phosphate-buffered saline and were assessed daily for disease severity. Survival was monitored for 2 weeks. Statistical comparison of susceptibility was done by Kaplan-Meier survival curve statistical analysis ($P<.001$).
was observed in infected WT mice by day 3 after infection, which was reduced substantially by day 5 after infection. 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 multilobed nuclei (Figure 4). By day 3 after infection, a substantially increased influx of cells was observed in large lesions, and by day 5 after infection, extensive foci of consolidation were visible, with massive accumulation of neutrophils around alveolar spaces (Figure 4). Flow cytometry of infiltrating cells in lungs confirmed that the majority of these cells were Ly6G+CD11b+ neutrophils (Figure 5A). The numbers of these cells were significantly higher in the infected Mincle−/− lungs than those in the WT mice (Figure 5A). This correlated with significantly higher levels of neutrophil chemoattractants (CXCL1 and CXCL6), neutrophil survival mediator (granulocyte macrophage colony-stimulating factor), and neutrophil activation markers (matrix metalloproteinase 9 and myeloperoxidase) in these mice, compared with levels in their WT counterparts (Figure 5B).

**Mincle−/− Neutrophils Are Defective in K. pneumoniae 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 *K. pneumoniae* was compared between WT and Mincle−/− neutrophils by flow cytometry. As shown in Figure 6, Mincle deficiency resulted in significantly reduced phagocytosis of nonopsonized bacteria by neutrophils. The uptake of opsonized bacteria was also reduced in Mincle−/− neutrophils, compared with Mincle-sufficient WT cells, but the differences were not statistically significant. These results indicate that Mincle is likely a novel nonopsonic phagocytic receptor for *K. pneumoniae* 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 NET-mediated killing has been shown to play a role in *K. pneumoniae* clearance [6, 34], we sought to determine whether Mincle deficiency resulted in a defect in NET formation. To minimize
tissue processing and avoid associated degradation of NETs, neutrophils isolated from bronchoalveolar lavage were used. Flow cytometry showed that neutrophils were a predominant cell type in the bronchoalveolar lavage of infected WT and Mincle$^{-/-}$ mice (Figure 7A). A quantitative comparison of bronchoalveolar lavage neutrophils showed that significantly higher numbers Mincle-sufficient WT neutrophils produced NETs (Figure 7B), which stained positive for neutrophil-specific enzyme neutrophil elastase (Figure 7C), showing that these fibrillar structures originated mainly from neutrophils.

Figure 3. Pneumonic Mincle$^{-/-}$ mice exhibit a hyperinflammatory response. The lungs from mock control and Klebsiella pneumoniae–infected wild-type (WT) and Mincle$^{-/-}$ mice were harvested at the indicated time points after infection, homogenized in phosphate-buffered saline with protease inhibitors, and analyzed commercially for host immune mediators, by a rodent multianalyte profile (Myriad RBM, Austin, TX). Results are average values for the infected and mock control groups (3–4 mice per group) in 3 independent experiments. The amounts of mediators shown were significantly higher (***$P < .001$) in $K$. pneumoniae–infected Mincle$^{-/-}$ mice 3 and 5 days after infection, compared with their levels in infected WT mice at the time points tested. Abbreviations: IFN-$\gamma$, interferon $\gamma$; IL-1$\beta$, interleukin 1$\beta$; IL-6, interleukin 6; IL-10, interleukin 10; TNF-$\alpha$, tumor necrosis factor $\alpha$.

Figure 4. Pneumonic Mincle$^{-/-}$ mice exhibit severe lung pathology characterized by massive accumulation of neutrophils. Hematoxylin and eosin staining of lung cryosections from mock control and Klebsiella pneumoniae–infected wild-type (WT) and Mincle$^{-/-}$ mice isolated at the indicated times after infection (original magnification, 100x). Inset shows a highly magnified area (original magnification, 1000x) of a lesion in an infected lung from a Mincle$^{-/-}$ mouse that depicts neutrophils, as indicated by characteristic multilobed nuclear morphology.
Furthermore, the NETs observed in Mincle−/− neutrophils appeared dwarfed and lacked the web-like appearance of NETs observed in WT mice. This observation, together with the reduced phagocytic ability of Mincle−/− neutrophils, shows that Mincle deficiency severely impairs neutrophil-mediated bacterial uptake and clearance mechanisms in lungs during pneumonic K. pneumoniae infection.

**DISCUSSION**

Pneumonic sepsis is a major healthcare burden worldwide, and *K. pneumoniae* is the most frequent Gram-negative bacterial 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 *K. pneumoniae* pneumonia. Here we report several novel findings. First, we are the first to show a clear phenotype in terms of a severely reduced survival rate of Mincle−/− mice upon pneumonic *K. pneumoniae* infection.

**Figure 5.** Increased neutrophil accumulation coincides with elevated expression of neutrophil chemoattractant and activation markers in lungs of *Klebsiella pneumoniae*–infected Mincle−/− mice. A, Flow cytometry of Ly6G+CD11b+ neutrophils in mock control and *K. pneumoniae*–infected wild-type (WT) and Mincle−/− mice. Total lung cells were isolated from mice by collagenase treatment 3 days after infection. The cells were stained with anti-Ly6G-APC and anti-CD11b-Pacific blue antibodies as markers for neutrophils. The bar graph shows the average of the total number of neutrophils in the lungs of 2–3 mock control and 3–4 *K. pneumoniae*–infected WT and Mincle−/− mice from 3 independent experiments. Dot plots shown on the right are from 1 representative experiment. Statistical significance is denoted by asterisks (**P < .001). B, The lungs from mock control and *K. pneumoniae*–infected WT and Mincle−/− mice were harvested at the indicated time points after infection and analyzed commercially for host immune mediators by a rodent multianalyte profile (Myriad RBM, Austin, TX). Levels of neutrophil chemoattractants (CXCL2, CXCL6, and granulocyte macrophage colony-stimulating factor [GM-CSF]) and activation markers (matrix metalloproteinase 9 [MMP-9] and myeloperoxidase [MPO]) are averages for infected and mock control mice (3–4 per group) from 3 independent experiments. Amounts of mediators shown were significantly higher (**P < .001) in Mincle−/− mice 3 and 5 days after infection, compared with levels in WT mice at the time points tested.

**Figure 6.** Mincle deficiency impairs neutrophil phagocytosis of non-opsonized bacteria. Peritoneal neutrophils from wild-type (WT) and Mincle−/− mice were incubated with green fluorescent protein–labeled *Klebsiella pneumoniae* with (opsonized) or without (nonopsonized) 10% normal mouse serum for 1 hour, followed by quantitation of phagocytosis by flow cytometry. The results are expressed as the percentage of cells positive for fluorescent bacteria. Significant differences are denoted by asterisks (**P < .005).
Second, the reduced survival is not due to a defect in ability to mount an inflammatory response in absence of Mincle. Third, Mincle acts as a nonopsonic phagocytic receptor mediating uptake of *K. pneumoniae* by neutrophils. Fourth, Mincle deficiency results in a defect in NET formation upon *K. pneumoniae* infection. Our findings thus show that Mincle is required for defense against *K. pneumoniae*-induced pneumonic sepsis and that the 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 the FcRγ-Syk-Card9 pathway to induce production of protective T-helper 1/T-helper 17 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 an increased bacterial and fungal burden, which was attributed to a reduced inflammatory response and defective pathogen clearance in these studies. However, the overall survival of the experimental animals was not affected by Mincle deficiency. Our study is the first to report a clear
outcome in which Mincle seems to play a nonredundant role in the survival of *K. pneumoniae*-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 an inflammatory response. It is likely that the redundant function of other PRRs, upon recognition of bacterial PAMPs and endogenous alarmin 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 and systemic bacterial burdens.

Concomitant with the increase in bacterial burden, Mincle−/− mice exhibited extensive accumulation of neutrophils, the primary cell type shown to play an important role in mediating the protective immune response against *K. pneumoniae* infection [4, 7, 18, 42]. We thus examined whether Mincle−/− neutrophils were defective in performing cellular functions such as the internalization of bacteria via phagocytosis, which would explain the increased bacterial burden in Mincle−/− mice despite heightened inflammation. Indeed, Mincle−/− neutrophils showed a mitigated phagocytosis of nonopsonized but not opsonized *K. pneumoniae*, suggesting a nonredundant and direct role of Mincle for internalization of nonopsonized bacteria. To the best of our knowledge, this is the first study to describe Mincle as a novel nonopsonic phagocytic receptor. Ongoing studies in our laboratory are currently investigating the Mincle-specific ligand of *K. pneumoniae*, the nature of this interaction, and the production of specific antibodies that can inhibit this interaction. Lectinophagocytosis, or lectin-mediated uptake by macrophages, has been reported previously for several pathogens [43]. However, the receptors or mechanisms of nonopsonized phagocytosis of bacteria by neutrophils are poorly understood. Nonopsonic 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 because serum and complement components are as such limited in the alveolar space [44]. Although the appearance of serum components in the alveolar space is common during severe *K. pneumoniae* pneumonia, owing to the high binding capacity of Mincle to mannose and N-acetylgalcosamine [45], uptake by Mincle of *K. pneumoniae* withmannan-rich capsule in the lungs could be a major mechanism of bacterial clearance in lungs. The absence of Mincle and a resulting defect in initial phagocytic uptake of *K. pneumoniae*, as observed in our studies, likely contributes to the increased bacterial burden and subsequent inflammation via activation of other PRRs such as Toll-like receptors.

One of the more recently defined mechanisms of antimicrobial activity of neutrophils is extrusion of the 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 *K. pneumoniae* 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 *K. pneumoniae*-induced pneumonia [6, 34]. How Mincle regulates NET formation is currently under investigation in our laboratory and is expected to provide novel insights into the mechanism of neutrophil NET release during cell death (ie, NETosis). This will have important implications in chronic disease conditions in which deregulated NET formation is associated with the pathophysiology.

Taken together, our results show that Mincle plays a protective role in *K. pneumoniae*-induced pneumonic sepsis by regulating neutrophil phagocytosis and NET formation, two important mechanisms of antimicrobial activity of neutrophils. In particular, the novel observation of Mincle as a potential new component of the NETosis pathway implicates this CLR in a much wider range of biological functions than initially surmised.

Notes

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