Collectin Kidney 1 Plays an Important Role in Innate Immunity against Streptococcus pneumoniae Infection

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Abstract
Collectins are C-type lectins that are involved in innate immunity as pattern recognition molecules. Recently, collectin kidney 1 (CL-K1) has been discovered, and in vitro studies have shown that CL-K1 binds to microbes and activates the lectin complement pathway. However, in vivo functions of CL-K1 against microbes have not been elucidated. To investigate the biological functions of CL-K1, we generated CL-K1 knockout (CL-K1\textsuperscript{−/−}) mice and then performed a \textit{Streptococcus pneumoniae} infection analysis. First, we found that recombinant human CL-K1 bound to \textit{S. pneumoniae} in a calcium-dependent manner, and induced complement activation. CL-K1\textsuperscript{−/−} mice sera formed less C3 deposition on \textit{S. pneumoniae}. Furthermore, immunofluorescence analysis in the wild-type (WT) mice demonstrated that CL-K1 and C3 were localized on \textit{S. pneumoniae} in infected lungs. CL-K1\textsuperscript{−/−} mice revealed decreased phagocytosis of \textit{S. pneumoniae}. Consequently, less \textit{S. pneumoniae} clearance was observed in their lungs. CL-K1\textsuperscript{−/−} mice showed severe pulmonary inflammation and weight loss in comparison with WT mice. Finally, the decreased clearance and severe pulmonary inflammation caused by \textit{S. pneumoniae} infection might cause higher CL-K1\textsuperscript{−/−} mice lethality. Our results suggest that CL-K1 might play an important role in host protection against \textit{S. pneumoniae} infection through the activation of the lectin complement pathway.

Introduction
Innate immune responses are the first defense line against invasive pathogens and are based on the recognition of microbial carbohydrate patterns on the cell surface. Collectins are a family of C-type lectins that are involved in host defense as pattern recognition molecules. Collectins share a common structure that is composed of a collagen-like domain, α-helical neck domain, and carbohydrate recognition domain (CRD). Collectins are assembled into trimers and further organized into higher structures that mediate recognition and phagocytosis of microorganisms.
oligomeric structures through the N-terminal cysteine-rich domain [1, 2].

Mannose-binding lectin (MBL) and surfactant protein A and D (SP-A, SP-D) were the first identified collectins. MBL is synthesized in the liver and expressed as a serum protein and makes a complex with MBL-associated serine proteases (MASPs) to activate the lectin complement pathway [3, 4]. MBL binds to microbes as an opsonin for phagocytes via the collectin receptor, and also acts as a viral neutralization factor [5, 6]. SP-A and SP-D are isolated from lung surfactant, and play important roles in host defense and lung homeostasis [7].

In the past decades, a number of studies have demonstrated that “novel collectin” collectin kidney 1 (CL-K1) plays a pivotal role in embryogenesis as well as in the innate immune system [8, 9]. The interaction of CL-K1 with sugar ligands shows a high affinity with high-mannose oligosaccharide composed of an α1–2 linkage [10]. Tissue distribution of CL-K1 was examined using RT-PCR, immunofluorescence, and immunohistochemistry, revealing mRNA and protein expression in the kidney, adrenal glands, small intestine, lung, liver, and brain [11]. CL-K1 in the blood is thought to be produced in hepatocytes in the liver. The blood concentration of CL-K1 in a healthy Japanese population group and Danish group was 0.34 and 0.28 μg/mL, respectively [12, 13]. The OD600 measured 0.6. Bacteria were heat-inactivated at 95°C for 10 min, then washed and resuspended by 0.1% BSA/PBS (phosphate-buffered saline, pH 7.2). CL-K1 was not treated with heat inactivation. Bacterial suspension was aliquoted and stored at −80°C until use.

Expression of Recombinant Human CL-K1

Recombinant human CL-K1 (rhCL-K1) was prepared as described previously [12]. Briefly, a stable CHO cell line that expressed full-length human CL-K1 was cultured for 7 days in a serum-free condition. The culture supernatant was incubated with mannan-agarose (Sigma-Aldrich, Japan) in the presence of 5 mM CaCl2, and rhCL-K1 was eluted by 0.1 M mannosyl/TBSC (Tris-buffered saline, 5 mM CaCl2, 0.1 M mannosyl). The eluted fraction was applied to PD-10 Desalting Column (GE Healthcare, Japan) to remove mannose just before using.

Materials and Methods

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Bacterial Strains and Culture Conditions

*S. pneumoniae* serotype 2 D39 strain (NCTC 7466) was grown on sheep blood agar plates for 20 h in a 5% CO2 incubator. *S. pneumoniae* was collected by sterilized cotton swabs, and were then resuspended in BHI (brain heart infusion) broth (Fluka, Japan). Escherichia coli serotype O6 (ATCC 25922) was grown in Luria-Bertani broth (Becton Dickinson, Japan) until the OD600 measured 0.6. Bacteria were heat-inactivated at 95°C for 10 min, then washed and resuspended by 0.1% BSA/PBS (phosphate-buffered saline, pH 7.2). *S. pneumoniae* was not treated with heat inactivation. Bacterial suspension was aliquoted and stored at −80°C until use.

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CL-K1-Binding Assay and Binding Inhibition Assay

An rhCL-K1- and native CL-K1-binding assay were mainly performed in a 1.5-ml centrifuge tube. rhCL-K1 (final concentration, 0.25, 2.5, 25 μg/mL) and normal human serum (0, 0.3, 1, 3, 10, 30%) as native CL-K1 were incubated with 10 μL of heat-inactivated bacteria (OD600 = 12) or zymosan (180 μg/mL) in 180 μL of 3% BSA/TBSC (TBSC, 0.1% Tween 20, 3% BSA) at 37°C for 1 h. For the inhibition assay, 15 mM ethylenediaminetetraacetic acid (EDTA, Wako, Japan), 0.1 M mannosyl (Nacalai Tasque, Japan), or 10 μg/mL Poly A or Poly I (Sigma-Aldrich, Japan) were added. After washing 3 times with TBSTC, rabbit anti-human CL-K1 polyclonal antibody [12] was added and incubated for 1 h at 37°C, followed by incubation with a HRP-conjugated secondary antibody and TMB Microwell Peroxidase substrate. The enzymatic reaction was stopped by adding 1 M H2PO4, and the tubes were centrifuged at 15,000 rpm for 5 min at 4°C to spin down microbes. Supernatants were transferred to a microtiter plate and the enzymatic reaction was measured at 450 nm using a microtiter plate reader. In the another binding analysis by the pull-down method, microbes treated with rhCL-K1 (final concentration, 25 μg/mL) were washed 5 times with TBSTC and resuspended in a protein sample buffer containing β-mercaptoethanol, then heated at 95°C for 5 min. The rhCL-K1 levels that bound to microbes and control rhCL-K1 were visualized by Western blotting with the same anti-CL-K1 polyclonal antibody.

Phagocytosis Assay

An in vitro phagocytosis assay was performed using a Vybrant Phagocytosis Assay Kit (Molecular Probes, USA) according to the manufacturer’s instructions. First, FITC-labeled *S. pneumoniae* was prepared. Briefly, freshly cultured *S. pneumoniae* was heat-inactivated at 95°C for 10 min and suspended in 0.1 M sodium bicarbonate (pH 9.0). *S. pneumoniae* was labeled with 10 μg/mL of...
FITC (Molecular Probes, USA) for 1 h in the dark. After washing with 0.1% BSA/PBS, it was aliquoted and stored at –80°C until use. 1 × 10⁶ cells/mL of mouse macrophage cell line J774A.1 was prepared and 100 μL of cell suspension was plated onto 96-well plates and incubated for 24 h at 37°C in a 5% CO₂ incubator. FITC-labeled E. coli (Molecular Probes, USA) and FITC-labeled S. pneumoniae were preincubated with or without of rhCL-K1 (25 μg/mL), and added to J774A.1 cells followed by incubation for 2 h at 37°C in a 5% CO₂ incubator. After removing the bacterial suspension, 100 μL of trypsin blue was added for quenching of the cell surface-bound FITC-labeled S. pneumoniae. This was followed by measuring fluorescence intensity using a fluorescence plate reader (~480 nm excitation, ~520 nm emissions).

**Mice**

CL-K1+/- mice were generated as described elsewhere [Mori et al., manuscript in preparation]. Genotyping of mice was performed using PCR with the following 3 primers, ON8053: 5’TCTGCTTCAAGCCATGAATCTCTGTTTGTA, ON8056: 5’CACGGGACAGGAAAGCCAGGCTAATCCAG, ON9093: 5’CTTGGGTGAGAGGCTATTCGGCTATGACT. CL-K1+/- mice were backcrossed into a C57BL/6J background more than 8 generations to obtain a pure genetic background. All mice were housed in specific pathogen-free conditions with an artificial 12-h dark-light cycle, with free access to standard food and drinking water. All experiments were carried out in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical University.

**Deposition of Mouse C3 by in vitro Assay**

Mice sera C3 deposition was assayed using ELISA. Nunc-Immuno™ MicroWell™ 96-Well Plates (Thermo Scientific, Japan) were coated with 10 μg/mL of zymosan or heat-inactivated S. pneumoniae D39 (OD₆₀₀ = 0.6) in a coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated overnight at 4°C. After washing with TBSTC, wells were blocked by TBS containing 0.1% human serum albumin for 1 h at 37°C. Serum was collected from 10 individual wild-type (WT) and CL-K1+/- male mice. 1% mice sera and serum (1%) was added to S. pneumoniae and incubated for 90 min at 37°C. After washing with TBSTC, rabbit anti-human C3c polyclonal antibody was added and incubated for 1 h at 37°C, followed by incubation with a secondary antibody and TMB Microwell Peroxidase substrate. The reaction was stopped by adding 1 M H₃PO₄, the tubes were centrifuged at 15,000 rpm for 5 min at 4°C to spin down S. pneumoniae. The supernatant was transferred to microtiter well plates and the enzymatic reaction was measured at 450 nm using a microtiter plate reader.

**Infection**

Male mice (12–15 weeks old) were anesthetized with pentobarbital, and 20 μL of BHI broth containing 0, 1 × 10⁵, 1 × 10⁶, or 1 × 10⁷ CFU (colony-forming units) of S. pneumoniae D39 was administered by pipetting into the nostrils. To determine the infection dose, a preliminary experiment was performed using WT mice. S. pneumoniae CFU in BHI broth were calculated as OD₆₀₀ = 38, which equals 1 × 10⁶ CFU/μL. For the survival investigation, visible clinical symptoms and body weight change were monitored for 10 days. The survival of the animal was assessed every 24 h.

**Lung CFU Calculation**

To examine the bacteria clearance of the lung, mice were infected intranasally with 1 × 10⁶ CFU of S. pneumoniae. At 48 h after infection, the mice were killed by cervical dislocation, and the lungs were collected into centrifugation tubes. Then the lungs were weighed and saline 4 times the volume of each lung was added. The lungs were homogenized and 100 μL of homogenate was mixed with 900 μL of sterilized saline. Serial dilution (10⁻⁶) was made with each lung sample, and 100-μL aliquots of each dilution sample were plated onto 5% sheep blood agar plates. The plates were incubated for 20 h at 37°C in 5% CO₂, and the number of CFU was counted.

**Histology**

Mice were infected intranasally with 1 × 10⁶ CFU of S. pneumoniae. At 48 h after infection, the mice were killed and the lungs were removed and fixed in a 10% formalin neutral buffer for 1 h. Four-micrometer paraffin-embedded sections were cut and placed onto microscope slides and stained with hematoxylin and eosin (HE). This was followed by a pathologist-blinded analysis of lung inflammation. Inflammation was scored using the following parameters: interstitial inflammation, intra-alveolar inflammation, edema, endothelitis, bronchitis, thrombosis formation, and pleuritis [19, 20]. Each parameter was graded on a scale of 0–3 as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. The total lung inflammation score was expressed as the sum of the scores for each parameter, so the maximum score was 21.

**Immunohistochemistry**

To detect CL-K1, S. pneumoniae, and C3 in infected mice lungs, WT and CL-K1+/- male mice were intranasally infected with 1 × 10⁶ CFU of S. pneumoniae. At 6 and 24 h after infection, the mice were killed and the lungs were removed and fixed in a 10% formalin neutral buffer solution for 24 h. Four-micrometer paraffin-embedded sections were cut and placed onto microscope slides, and deparaffinized by a series of methanol baths.

To improve immunoreactivity, sections were treated with an antigen retrieval buffer (Citrate Buffer pH 6.0, Dako, Japan), and heated in a microwave. After washing with PBS, sections were blocked for endogenous avidin and biotin by incubating with avian.
din solution for 15 min followed by biotin solution for 15 min (Avidin/Biotin-Blocking System, Dako, Japan). Then sections were blocked with Block Ace (Dainippon Pharmaceutical, Japan) for 1 h at room temperature followed by incubation with either affinity-purified rabbit anti-CL-K1 polyclonal antibody [11], rabbit anti-human C3c polyclonal antibody, or control IgG for 20 h at 4 °C. After primary antibody incubation, these sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies, USA) for 1 h at room temperature. For S. pneumoniae, the above slides were incubated with biotin-conjugated rabbit anti-S. pneumoniae polyclonal antibody (ViroStat, USA) for 2 h at 4 °C. After washing, slides were incubated with Alexa Fluor 555 streptavidin (Life Technologies, USA) for 1 h at room temperature followed by mounting in SlowFade Gold Antifade Reagent (Molecular Probes, USA). Serial sections were stained with HE to visualize lung tissue. The fluorescence images were obtained using a confocal laser-scanning microscope (FV1000; Olympus Optical, Japan).

**In vivo Phagocytosis Assay**

WT and CL-K1–/– mice were infected intranasally with 1 × 10^6 CFU of S. pneumoniae. At 6 h after infection, the mice were killed and the lungs were removed and fixed in a 10% formalin neutral buffer for 24 h. Four-micrometer paraffin-embedded sections were cut and placed onto microscope slides and stained with biotin-conjugated rabbit anti-S. pneumoniae antibody followed by AlexaFluor 555 streptavidin. Over 100 cells were counted on several microscopic fields and the phagocytic index was expressed as the average number of AlexaFluor 555-stained S. pneumoniae per single phagocyte.

**Statistical Analysis**

Statistical analysis was performed using the Student t test included in the JMP statistics software package (Version 7, SAS, Japan). Statistical analysis of the survival rate was performed using a log-rank test. *p < 0.05 was considered statistically significant.

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**Fig. 1.** CL-K1–/– mice showed increased susceptibility to S. pneumoniae infection. **a** The first experiment was performed to identify the LD_{50} of WT mice. WT mice were intranasally infected with 0, 1 × 10^5, 1 × 10^6, 1 × 10^7, or 3 × 10^7 CFU of S. pneumoniae (n = 6 in each group), and consequently the LD_{50} was calculated by their survival rates. **b–d** WT and CL-K1–/– mice were intranasally infected with 1 × 10^7 CFU of S. pneumoniae and the survival rates were calculated. CL-K1–/– male mice (n = 9) showed increased mortality compared with WT mice (n = 11), *p = 0.0163 for the log-rank test (b). CL-K1–/– female mice (n = 11) also showed increased mortality compared with WT mice (n = 22), *p = 0.0258 for the log-rank test (c). Total CL-K1–/– mice (n = 20) showed increased susceptibility compared with WT mice (n = 33), *p = 0.0012 for the log-rank test (d). *p < 0.05.
CL-K1 Is Related to the Resistance to S. pneumoniae Infection

Results

CL-K1−/− Mice Are More Susceptible to S. pneumoniae Infection

Male WT mice were infected with 0, 1 × 10^5, 1 × 10^6, 1 × 10^7 CFU of S. pneumoniae (Fig. 1a). All mice infected with 0, 1 × 10^5, or 1 × 10^6 CFU survived until 10 days after infection. In contrast, all mice infected with 3 × 10^7 CFU died within 3 days after infection. 1 × 10^7 CFU-infected mice began to die at 4 days after infection and 30% of the mice survived over the entire experimental period (10 days). Based on these results, we performed sequential and histological lung analyses (0, 6, and 24 h post infection) in WT and CL-K1−/− mice infected with 1 × 10^6 CFU of S. pneumoniae. At 6 h after infection, WT and CL-K1−/− mice lungs showed similar levels of inflammation. However, at 24 h after infection, CL-K1−/− mice lungs showed more severe inflammation compared to WT mice lungs (Fig. 4, HE). CL-K1 immunofluorescence staining in WT mice lungs demonstrated that a high expression of CL-K1 was observed at 6 h after infection and remained until 24 h after infection in bronchial and alveolar epi-

Clearance of S. pneumoniae Is Decreased in CL-K1−/− Mice

We examined murine lung CFU and body weights at 48 h after 1 × 10^6 CFU of S. pneumoniae inoculation. Greater numbers of viable S. pneumoniae were observed in CL-K1−/− mice lungs compared with those in WT mice lungs (Fig. 2a, WT, 20,425 ± 20,650; CL-K1−/−, 90,616 ± 59,395, p < 0.005). The body weight of WT mice decreased 6% at 48 h after infection and weights of CL-K1−/− mice decreased 9% at the same time (Fig. 2b, p < 0.05). These results suggest that the lower clearance of S. pneumoniae in CL-K1−/− mice might cause severe harm to the mice.
The staining of CL-K1−/− mice lungs was negative (Fig. 4, CL-K1). In S. pneumoniae staining, a large number of S. pneumoniae was observed in lungs of CL-K1−/− mice at 6 and 24 h after infection, although the numbers decreased in the lungs of WT mice at 24 h after infection (Fig. 4, S. pneumoniae). We already observed that same CFU (1 × 10⁶)-infected CL-K1−/− mice at 48 h after infection showed a greater number of bacteria and more severe weight loss compared with WT mice (Fig. 2a, b).

**CL-K1 Directly Interacts with S. pneumoniae and Activates the Lectin Pathway**

To determine the binding ability of CL-K1 to S. pneumoniae serotype 2 D39 strain, we incubated S. pneumoniae for 1 h with normal human serum (final concentration of 0, 0.3, 1, 3, 10, 30%) and rhCL-K1 (0, 0.25, 2.5, 25 μg/mL). The bound CL-K1 was visualized by an ELISA system (Fig. 5a, b) and Western blotting (Fig. 5c, d). The ELISA system using rhCL-K1 and normal human serum demonstrated that both bindings between bacteria and
rhCL-K1 or native CL-K1 were dose dependent and were found in the physiological condition concentration of 0.25 μg/mL. We also confirmed the similar binding activity in viable and heat-inactivated bacteria (online suppl. Fig. 2). Next we examined the binding ability of rhCL-K1 to gram-negative bacteria *E. coli* serotype O6 (S type LPS), and fungus cell wall extract zymosan. It was found that rhCL-K1 interacted with *E. coli* and zymosan (online suppl. Fig. 3). We found that rhMBL only recognized and interacted with zymosan, while no binding activity was observed in *S. pneumoniae* and *E. coli* in the same assay system (data not shown). These results are consistent with previous studies that have demonstrated the interaction of MBL with microbes [21–23].

CL-K1 is considered to interact with specific complex carbohydrates in a calcium-dependent manner via the
In this study, we performed the inhibition analysis using several inhibitors to identify the binding specificities of CL-K1 against microbes. We found that EDTA and mannose inhibited partially the binding of rhCL-K1 to *S. pneumoniae* (Fig. 5, Table 1, 26 ± 4%, *p* < 0.005 and 43 ± 26%, *p* < 0.05, respectively). These results indicate that CL-K1 recognizes carbohydrate patterns on *S. pneumoniae* cell surfaces in a calcium-independent manner via the CRD. Interestingly, positive charge inhibitor Poly A also strongly inhibited the binding to *S. pneumoniae* (1 ± 2%, *p* < 0.005).

Another binding inhibition assay revealed that CL-K1 interacted with *E. coli* in a calcium-independent manner (online suppl. Fig. 3). EDTA did not inhibit the binding to *E. coli*. However, mannose inhibited the

**Table 1.** rhCL-K1-binding rate (%) of *E. coli*, zymosan, and *S. pneumoniae*  

| Microbes       | CL-K1 binding, % of control | EDTA (15 mM) | Mannose (0.1 M) | Poly A (10 μg/mL) | Poly I (10 μg/mL) |
|----------------|----------------------------|--------------|-----------------|-------------------|------------------|
| *E. coli*      | 100                        | 113±71       | 41±26*          | 62±27             | 229±110          |
| Zymosan        | 100                        | 29±6**       | 19±11**         | 93±10             | 134±53           |
| *S. pneumoniae*| 100                        | 26±4**       | 43±26*          | 1±2*              | 54±33            |

Data represent means ± SD of 3 independent experiments. *p* < 0.05; **p* < 0.005.
binding to *E. coli*. On the other hand, in case of the fungus, EDTA and mannose significantly inhibited the binding to zymosan (online suppl. Fig. 3). These results suggest that CL-K1 binds to zymosan in a calcium-dependent manner via the CRD like MBL. Our in vitro binding analyses suggest that rhCL-K1 might be able to interact directly with several microbes in a calcium- and charge-dependent manner.

To investigate the complement activation by CL-K1 on *S. pneumoniae*, C3 deposition analysis was performed using rhCL-K1. Human serum was passed through a protein G column to remove anti-*S. pneumoniae* antibodies [24]. To prevent lectin pathway activation, the above serum was treated with GlcNAc agarose beads to eliminate ficolin [25]. Analysis using double-depleted serum demonstrated that C3 deposition significantly increased following before incubation of rhCL-K1 (Fig. 6a). These results indicate that CL-K1 directly binds to *S. pneumoniae* and activates the complement system. Next, we examined the complement activity of CL-K1 before incubation of *S. pneumoniae* (Fig. 6b). These data indicate that rhCL-K1 might act as an opsonin against *E. coli*, but not act against *S. pneumoniae* in vitro.

**CL-K1 Interacts with S. pneumoniae in WT Mice Lungs and CL-K1−/− Mice Showed Decreased Phagocytosis of S. pneumoniae**

To elucidate the interaction between CL-K1 and *S. pneumoniae* in murine lungs, WT and CL-K1−/− mice were intranasally infected with 1 × 10^6 CFU of *S. pneumoniae*. In the early phase of infection, we observed *S. pneumoniae* colocalized with CL-K1 and counted the number of *S. pneumoniae* engulfed by phagocytes in WT (*n* = 2) or CL-K1−/− (*n* = 2) mice lungs (Fig. 7c, d). The numbers of *S. pneumoniae* particles phagocytosed by one phagocyte were significantly lower in CL-K1−/− mice compared to those in WT mice (4.11 ± 2.07 in WT mice and 3.03 ± 1.75 in CL-K1−/− mice, *p* < 0.001). C3 deposits were also observed on engulfed *S. pneumoniae*, although we could not perform the double staining by anti-CL-K1 and anti-C3 polyclonal Ab. These data suggest that CL-K1 might be able to activate the complement pathway in murine lungs, thereby augmenting the phagocytosis of *S. pneumoniae*.

**rhCL-K1 Exhibits Opsonic Activity against E. coli but Not against S. pneumoniae**

To assess the opsonic activity of rhCL-K1 against *S. pneumoniae*, we carried out a phagocytosis analysis. FITC-labeled *E. coli* or *S. pneumoniae* were preincubated with or without rhCL-K1 (25 μg/mL), and then incubated with the mouse macrophage cell line J774A.1. As shown in Figure 7a, we could observe a significant increase in the phagocytosis of *E. coli* K12 after preincubation of rhCL-K1. However, preincubation of rhCL-K1 did not influence *S. pneumoniae* phagocytosis (Fig. 7b). These data indicate that rhCL-K1 might act as an opsonin against *E. coli*, but not act against *S. pneumoniae*.
Discussion

Collectins and ficolins generally play a role in opsonization, complement activation, and the elimination of microbes against invading pathogens [1, 2, 25]. Among these biological functions, complement activation is considered to be particularly important in protecting the body from infectious diseases [25, 26]. Furthermore, it was found that complement factors were present in the lung which might activate complement system during bacterial infection [25, 27]. The components of C1q, factor B, C2, C3, C4, C5, and C6 in humans, and C3, C5, and factor B in mice and rats were observed in bronchoalveolar lavage [27]. However, it has not been shown that CL-K1 is present in bronchoalveolar lavage.

In this study, we investigated the host defense roles of CL-K1 against S. pneumoniae with in vitro and in vivo analyses. Recent studies indicate that CL-K1 in human and mouse serum might interact with S. pneumoniae [28]. However, direct interaction between CL-K1 and S. pneumoniae has not been demonstrated clearly. Here we found that rhCL-K1 directly interacted with S. pneumoniae in a charge-dependent manner in addition to being due to calcium-dependent lectin activity (Fig. 5c, d; Table 1). We consider that CL-K1 has a positively charged region in its structure and that it can additionally interact with negatively charged S. pneumoniae.

Recently, CL-K1 has been shown to bind to DNA ligands in a charge-dependent manner, but not in a calcium-dependent manner [29]. Two well-known collectins, MBL and SP-D, can also bind DNA by their CRDs and collagen-like regions, but not strongly [30, 31].

Online supplementary Figure 3 and Table 1 show that the binding mechanism of rhCL-K1 to E. coli and zymosan was different from that of S. pneumoniae. The reason is because the interactions between rhCL-K1 to E. coli
and zymosan were not inhibited by any charged ligands. We demonstrated that rhCL-K1 interacted with E. coli due to calcium-independent lectin activity and that it interacted with zymosan due to calcium-dependent lectin activity, respectively, via CRD. These results suggest that CL-K1 might utilize 2 unique domains of the CRD-binding site and the collagen-like region to recognize and interact with various microbes, although further study using a deletion mutant of the collagen-like region or CRD is needed.

In previous studies, sandwich ELISA using human EDTA plasma showed the interaction between CL-K1 and MASPs, and rhCL-K1 bound to C. albicans and induced the deposition of C4b, C3b, and MAC on the surface of C. albicans [15, 16]. Our C3 deposition analysis demonstrated that CL-K1 might cause the activation of the lectin complement pathway on the surface of S. pneumoniae (Fig. 6a). It is well known that microbes opsonized by complement are easily recognized by phagocytes, which express complement receptors on their surface, and consequently lead to increased phagocytosis [26]. In in vitro analysis, rhCL-K1 could increase phagocytosis of E. coli, but it did not enhance the phagocytosis of S. pneumoniae (Fig. 7a, b). In murine experiments, C3 deposition on S. pneumoniae by CL-K1<sup>−/−</sup> mice sera was lower than WT mice sera, and CL-K1<sup>−/−</sup> mice showed a greater number of S. pneumoniae in the lungs compared to WT mice after inoculation (Fig. 6b, 2a). These results suggest that CL-K1 in animal models might be able to affect complement activation and reduce the number of microbes. The detailed mechanism of the biological function in CL-K1 in vivo needs to be resolved in the future.

In the murine infection study, we observed that S. pneumoniae infection of CL-K1<sup>−/−</sup> mice caused more severe inflammation in their lungs compared to WT mice (Fig. 3). A previous study reported that MASP-2<sup>−/−</sup> mice infected with S. pneumoniae exhibited increased proinflammatory cytokines (e.g., TNFa and IL-6) and these mRNA expressions led to severe inflammation in the lungs [28]. Recently, it was reported that neutrophil extracellular trap formation plays an important role in inflammation after microbe infection. Neutrophils can catch DNA from cells and broken microbes and regulate the inflammation [32, 33]. The presence of neutrophil extracellular trap formation has been demonstrated in various inflammatory lung diseases including asthma, cystic fibrosis, and microbe infections. It has been shown that CL-K1 binds just as strongly to nucleotides as DNA compared to polysaccharide [29]. It indicates that CL-K1 might be able to bind these DNA during/after pulmonary microbial infection and might be related to neutrophil extracellular trap-related inflammation due to complement activation.

WT and CL-K1<sup>−/−</sup> mice showed almost the same amount of S. pneumoniae in the lungs at 6 h after infection. However, at 24 h after infection, WT mice demonstrated lower amounts of S. pneumoniae than CL-K1<sup>−/−</sup> mice (Fig. 4). CL-K1<sup>−/−</sup> mice showed larger amounts of S. pneumoniae even at 48 h after infection compared with WT mice (Fig. 2a). We believe that the reduced survival rate of CL-K1<sup>−/−</sup> mice after S. pneumoniae infection might be due to bacteria proliferation and severe pulmonary inflammation (Fig. 1b–d).

Mutations in both CL-K1 and MASP-3 coding genes is associated with 3MC (Mingarelli, Malpuech, Michels, and Carnevale) syndrome. This congenital disorder is characterized by facial dysmorphism, cleft palate, craniosynostosis, learning disabilities, and genital, limb, and vesicorenal anomalies [9]. Three separate mutations in CL-K1 are associated with 3MC syndrome. In transient transfection studies, these CL-K1 mutant proteins were not secreted into the medium [10]. This fact indicates that the secretion of CL-K1 might play a crucial role not only in innate immunity, but also in fetal development. Our results in murine infection experiments suggest that 3MC syndrome patients might show innate immunity dysfunction, although this has not been reported yet.

In summary, CL-K1 binds directly to S. pneumoniae and increases opsonophagocytosis. CL-K1 might also activate the lectin complement pathway, and consequently reduce numbers of S. pneumoniae in murine-infected lungs. CL-K1 deficiency shows increased lethality from S. pneumoniae infections. Finally, our study demonstrated the host-protecting role of CL-K1 against S. pneumoniae infection.

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Disclosure Statement

The authors declare that they have no financial conflicts of interest.
References

1. Sastry K, Ezekowitz RA: Collectins: pattern recognition molecules involved in first line host defense. Curr Opin Immunol 1993;5:59–66.
2. Holmskov U, Jenseniussen JC: Structure and function of collectins: humoral C-type lectins with collagenous regions. Behring Inst Mitt 1993;93:224–235.
3. Kawasaki N, Kawasaki T, Yamashina I: Isolation and characterization of a mannan-binding protein from human serum. J Biochem 1983;94:937–947.
4. Matsushita M, Fujita T: Activation of the classical complement pathway by mannos-binding protein in association with a novel C1s-like serine protease. J Exp Med 1992;176:1497–1502.
5. Malhotra R, Thiel S, Reid KBM, Sim RB: Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J Exp Med 1990;172:955–959.
6. Kase T, Suzuki Y, Kawai T, Sakamoto T, Ohtani K, Eda S, Maeda A, Okuno Y, Waka-miya N: Human mannan-binding lectin inhibits the infection of influenza A virus without complement. Immunology 1999;97:385–392.
7. Kuroki Y, Takahashi M, Nishitani C: Pulmonary collectins in innate immunity of the lung. Cell Microbiol 2007;9:1871–1879.
8. Keshi H, Sakamoto T, Kawai T, Ohtani K, Kato H, Jang S, Motomura W, Yosihashi T, Fukuda M, Koyama S, Fukuzuka J, Fukuh A, Yoshida I, Suzuki Y, Wakamiya N: Identification and characterization of a novel human collectin CL-K1. Microbiol Immunol 2006;50:1001–1013.
9. Rooryck C, Diaz-Font A, Osborn DPS, Chabchoub E, Hernandez-Hernandez V, Sham-el din H, Kenny J, Waters A, Jenkins D, Kais-si AA, Leaf GF, Dallapiccola B, Peeters H, Al-kuraya FS, Beales P: Mutation in lectin pathway genes COLLEC11 and MASP1 cause 3MC syndrome. Nat Genet 2011;43:197–203.
10. Girija UV, Furze CM, Gingras AR, Yosihashi T, Ohtani K, Marshall JE, Wallis AK, Schwaeuble WJ, El-Mezgueldi M, Mitchell DA, Moody T, Ohtani K, Marshall JE, Wallis AK, Schwaeuble WJ, Dieter D, Girija UV, Wallis RW, Radioglu A, Stover CM, Andrew PW, Schwaeuble WJ: The lectin pathway of complement activation is a critical component of innate immune response to pneumococcal infection. PLoS Pathog 2012;8:e1002793.
11. Henriksson ML, Brandt J, Huang S, Nomura N, Suzuki/Yawida I, Suzuki Y, Kohgo Y, Mori K, Kitamoto N, Yoshida I, Suzuki Y, Wakamiya N: Concentrations of collectin kidney 1 and mannan-binding lectin. J Biochem 2012;151:57–64.
12. Selman L, Henriksson ML, Brandt J, Palarasah Y, Waters A, Beales PL, Holmskov U, Jorgensen TJ, Nielsen C, Skjoldt K, Hansen S: An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1). J Immunol Methods 2012;31:182–188.
13. Takahashi K, Ohtani K, Larvie M, Moyo P, Chigwelesi E, Van Cott EM, Wakamiya N; Elevated plasma CL-K1 level is associated with a risk of developing disseminated intravascular coagulation (DIC). J Thromb Thromboly-sis 2014;38:331–338.
14. Ma YJ, Skjoldt MO, Garred P: Collectin-11/MAFP complex formation triggers activation of the lectin complement pathway – the fifth lectin pathway initiation complex. J Innate Immun 2013;5:242–250.
15. Hansen S, Selman L, Palaniyar N, Ziegler K, Brandt J, Kleim A, Jonasson M, Skjoldt M, Nielsen O, Hartshorn K, Jorgensen TJ, Skjoldt K, Holmskov U: Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. J Immunol 2010;185:6096–6104.
16. Henriksson ML, Brandt J, Andrue J, Nielsen C, Jensen PH, Holmskov U, Jorgensen TJ, Palarasah Y, Thielens NM, Hansen S: Hetero-meric complexes of native collectin kidney 1 and collectin liver 1 are found in the circulation with MASPs and activate the complement system. J Immunol 2013;191:6117–6127.
17. Jeong D, Jeong E, Seo J, Heo S, Choi Y: Difference in resistance to Streptococcus pneumoniae infection in mice. Lab Anim Res 2011;27:91–98.
18. Boelen A, Kwakkel J, Wieland CW, Stap J, Kliem A, Jonasson M, Skjodt M, Lindberg MJ, Kliem A, Jonasson M, Skjodt M, Lindberg MJ, Furtner T, Hainzl E, Elbau I, Mesteri I, Doninger B, Binder RB, Knapp S: Myeloid PTEN deficiency causes 3MC syndrome. Nat Genet 2002;39:16969–16974.
19. Endo Y, Takahashi M, Iwaki D, Ishida Y, Nakazawa N, Kodama T, Matsuizaka T, Kanno K, Liu Y, Tsuchiya K, Kawamura I, Ikawa M, Waguri S, Wada I, Matsushita M, Shwaebel WJ, Fujita T: Mice deficient in ficolin, a lectin complement pathway recognition molecule, are susceptible to Streptococcus pneumoniae infection. J Immunol 2012;189:5860–5866.
20. Bolger MS, Ross DS, Jiang H, Frank MM, Ghio AJ, Schwartz DA, Wright JR: Complement levels and activity in the normal and LPS-injured lung. Am J Physiol Lung Cell Mol Physiol 2007;292:748–759.
21. Ali YM, Lynch NJ, Halsem KS, Fujita T, Endo Y, Hansen S, Holmskov U, Takahashi K, Stahl GL, Dudler T, Girija UV, Wallis RW, Radioglu A, Stover CM, Andrew PW, Schwaeuble WJ: The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. PLoS Pathog 2012;8:e1002793.
22. Henriksson ML, Brandt J, Lyer SSC, Thielens NM, Hansen S: Characterization of the interaction between collectin 11 (CL-11, CL-K1) and nucleic acids. Mol Immunol 2013;56:757–767.
23. Hakansson K, Reid KB: Collectin structure: a review. Protein Sci 2000;9:1607–1617.
24. Palaniyar N, Nadesalingam J, Clark H, Shih MJ, Dodds AW, Reid KB: Nucleic acid is a novel ligand for innate, immune pattern recognition collects surfactant proteins A and D and mannos-binding lectin. J Biol Chem 2004;279:32728–32736.
25. Knight JS, Carmona-Rivera C, Kaplan MJ: Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. Front Immunol 2012;3:380.
26. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner WD, Steinbacher P, Winterberg N, Bathke AC, Klappacher M, Studnicka M: Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. Respir Res 2015;16:59.