CD44 Deficiency Is Associated with Increased Bacterial Clearance but Enhanced Lung Inflammation During Gram-Negative Pneumonia

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***Klebsiella pneumoniae* is a frequently isolated causative pathogen in respiratory tract infections. CD44 is a transmembrane adhesion molecule that has been implicated in several immunological processes. To determine the role of CD44 during *Klebsiella* pneumonia, we intranasally infected wild-type and CD44 knockout (KO) mice with 10² to 10⁴ colony-forming units of *K. pneumoniae* or administered *Klebsiella* lipopolysaccharide. During lethal infection, CD44 deficiency was associated with reduced bacterial growth and dissemination accompanied by enhanced pulmonary inflammation. After infection with lower *Klebsiella* doses, CD44 KO mice but not wild-type mice demonstrated mortality. After infection with even lower bacterial doses, which were cleared by most mice of both strains, CD44 KO mice displayed enhanced lung inflammation 4 and 10 days postinfection, indicating that CD44 is important for the resolution of pulmonary inflammation after non-lethal pneumonia. In accordance, CD44 KO mice showed a diminished resolution of lung inflammation 4 days after intrapulmonary delivery of lipopolysaccharide. CD44 deficiency was associated with the accumulation of hyaluronan together with reduced gene expression levels of the negative regulators of Toll-like receptor signaling, interleukin-1R-associated kinase M, A20, and suppressor of cytokine signaling 3. In conclusion, the absence of CD44 affects various components and phases of the host response during *Klebsiella* pneumonia, reducing bacterial outgrowth and**

dissemination and enhancing pulmonary pathology during lethal infection, and diminishing the resolution of lung inflammation during sublethal infection. (Am J Pathol 2010; 177:2483–2494; DOI: 10.2353/ajpath.2010.100562)

Pneumonia is a common and serious illness that is a major cause of morbidity and mortality. *Klebsiella* (*K.*) *pneumoniae* is a frequently isolated causative pathogen in lower respiratory tract infection.^{1–3} The high incidence of pneumonia and the increasing resistance of respiratory pathogens to antimicrobial agents stress the importance of gaining more insight into the pathogenesis of this infection.^{1,4}

CD44 is a transmembrane adhesion molecule known to be involved in binding and metabolism of hyaluronan (HA).^{5,6} CD44 is present on a wide variety of cell types, including leukocytes and parenchymal cells. It has several functions in innate and adaptive immune responses such as involvement in cellular adhesion and migration, activation and proliferation of lymphocytes and monocytes, and cell mediated cytotoxicity in NK cells.⁷ In accordance, CD44 has been shown to play an important role in the (sub)acute inflammatory response to both infectious and sterile stimuli.^{8–11} CD44 is also involved in the resolution of inflammation as demonstrated by *in vivo* models of noninfectious lung injury; on bleomycin administration CD44 deficiency resulted in prolonged accumulation of inflammatory cells and a persistent rise in HA levels,¹² and on intrapulmonary delivery of lipopolysaccharide (LPS) CD44 deficiency resulted in a proinflammatory phenotype as shown by prolonged nuclear factor κ B activation in the lung.⁸ Furthermore, during infection CD44 may influence host defense by affecting phagocytosis. For instance, phagocytosis of *Staphylococcus* (*S.*) *aureus* is induced on activation of CD44 on human neutrophils by HA or monoclonal antibodies,¹³ whereas our laboratory

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showed that CD44 on murine macrophages mediates phagocytosis of *Mycobacterium (M.) tuberculosis*.¹⁴

To the best of our knowledge, only one study investigated the role of CD44 during bacterial pneumonia, focusing on the acute response during respiratory tract infection caused by *Escherichia (E.) coli* or *Streptococcus (S.) pneumoniae*.⁹ This investigation demonstrated an enhanced inflammatory response in lungs of CD44 knock-out (KO) mice 6 hours after infection with *E. coli*, as reflected by an increased influx of neutrophils and more edema, whereas the pulmonary response to *S. pneumoniae* was not influenced by CD44 deficiency.⁹ The role of CD44 during more prolonged lung infection (ie, beyond 6 hours) was not studied in either model.⁹ Therefore, in the present study we sought to investigate the function of CD44 during *K. pneumoniae* pneumonia, comparing both the induction of the acute innate immune response and the resolution of inflammation after lethal and sublethal infection in CD44 KO and wild-type (WT) mice.

Materials and Methods

Mice

Eight- to 12-week-old C57BL/6 WT mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). CD44 KO mice backcrossed >8× on a C57BL/6 background (kindly provided by Dr. A. Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands¹⁵) were bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands).

Study Design

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced by intranasal inoculation of 10² to 10⁴ colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Rockville, MD), as described previously (*n* = 8–12 per group).^{16–18} In indicated experiments *K. pneumoniae*-derived LPS (L1519, Sigma, St. Louis, MO; 1 μg) was administered intranasally (*n* = 8 per group). Sample harvesting and processing, and determinations of bacterial loads and cell counts were done as described.^{17,19} In one experiment mice (*n* = 9 per group) were given 10 mg/kg subcutaneously hyaluronan-binding peptide (HABP: RGGGRGRRR) or scrambled binding peptide control (scr: RGRGRGRG) (Invitrogen, Glasgow, UK) 1 hour before infection.^{20,21} For survival studies mice (*n* = 12 per group) were monitored for 10 days after infection.

Histopathology

Lungs were harvested after indicated time points, fixed in 10% buffered formalin, and embedded in paraffin. Hematoxylin and eosin-stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis and percentage of the

lung surface demonstrating confluent (diffuse) inflammatory infiltrate by a pathologist blinded for groups.^{17,19} The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 28. Granulocyte staining was performed using fluorescein isothiocyanate-labeled anti-mouse Ly-6G mAb (PharMingen, San Diego, CA) as described earlier.^{22,23} Ly-6G-stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzlar, Germany). Ten random pictures were taken per slide. Colored areas were analyzed with Image Pro Plus (Media Cybernetics, Bethesda, MD) and expressed as percentage of the total surface area. The average of 10 pictures was used for analysis.

Assays

Tumor necrosis factor-α, interleukin (IL)-6, IL-1β, keratinocyte-derived cytokine, MIP-2, LPS-induced CXC chemokine, and myeloperoxidase were measured by enzyme-linked immunosorbent assay using matched antibody pairs according to the manufacturer's instructions (myeloperoxidase from Hycult, Uden, The Netherlands; others from R&D Systems, Abingdon, UK).

mRNA Expression Analysis

RNA was isolated from lung homogenates that were immediately dissolved in TRIzol (Invitrogen, Breda, the Netherlands), as described by the manufacturer and reverse transcribed using oligo dT (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Reverse-transcription polymerase chain reactions were performed using LightCycler SYBR Green I Master Mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus using the following conditions: 5-minute 95°C hot-start, followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 5 seconds, 72°C for 15 seconds). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA, and data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression of the housekeeping gene β2-microglobulin (B2M).²⁴ Primers were as follows: β2-microglobulin, 5'-TG-GTCTTCTGGTGCTTGCT-3' and 5'-ATTTTTCCTTCTTCAGC-3'; A20, 5'-GGGACTCCAGAAAACAAGGG-3' and 5'-TACCCTTCAAACATGGTGCTT-3'; interleukin-1R-associated-kinase (IRAK)-M, 5'-TGCCAGAAGAATACAT-CAGACAG-3' and 5'-TCTAAGAAGGACAGGCAGGAGT-3'; suppressor of cytokine signaling (SOCS)3, 5'-ACCTT-TCTTATCCGCGACAG-3' and 5'-TGCACCAGCTTGAGT-ACACAG-3'.

Hyaluronan Measurements

HA levels in bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (R&D Systems). For HA molecular weight determination lung tissues were cut into fine pieces and digested in 500 μl of 0.15 mol/L NaCl containing 200 U/ml protease (Calbio-

chem, Darmstadt, Germany) at 55°C for overnight. The enzyme was then inactivated by boiling the samples at 100°C for 10 minutes. Samples were centrifuged at 5000 rpm for 5 minutes. HA-containing supernatants were concentrated with centrifugal filter devices (cutoff size 5 kDa) to get the final volume around 100 μ l. HA concentration of the samples was measured by HA enzyme-linked immunosorbent assay.¹² Two μ g of HA from each sample was aliquoted and brought to equal volume by adding 0.15 mol/L NaCl for gel electrophoresis. HA samples and standards were run on 0.5% agarose gel. HA gels were stained in 50% alcohol containing 0.005% Stains-All (Sigma, St. Louis, MO) for overnight under light protection at room temperature. The HA gels were then destained in water under light protection at room temperature for 2 days. Final destaining occurs on exposure to ambient light for approximately 30 minutes. HA gel images were documented using Geliance 600 imaging system from PerkinElmer. The captured agarose gel images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD). A standard curve was determined by the running distance of known molecular mass HA standards. HA peaks in the samples were calculated against the standard curve.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Comparisons between groups were analyzed using Mann-Whitney *U*-test. Survival was compared by log-rank test. A value of $P < 0.05$ was considered to represent a statistically significant difference.

Results

CD44 Deficiency Limits Bacterial Outgrowth and Dissemination in Severe Pneumonia

We first sought to determine the role of CD44 in host defense during severe *Klebsiella* pneumonia. For this we infected CD44 KO and WT mice with 10^4 CFU of *K. pneumoniae*, a bacterial dose that causes pulmonary

Figure 1. CD44 deficiency does not impact survival after infection with a lethal dose of *Klebsiella*. WT (closed symbols) and CD44 KO (open symbols) mice were intranasally infected with 10^4 CFU of *K. pneumoniae* and followed for 1 week. $n = 12$ mice per group. The data presented are derived from one single experiments in which all animals were infected at the same time. P value indicates no significant difference between groups.

sepsis and lethality in normal immunocompetent mice.^{16,18} To obtain a first insight into the impact of CD44 deficiency in this severe model, we conducted an observational study, following CD44 KO and WT mice for 1 week after infection. In both groups the first deaths occurred after 2 days; although CD44 KO mice tended to succumb later than WT mice, the difference between strains was not significant (Figure 1). To examine whether CD44 deficiency influences local bacterial outgrowth and

Figure 2. CD44 KO mice display decreased bacterial outgrowth during lethal *Klebsiella* pneumonia. Bacterial loads determined in lung (A), blood (B), spleen (C), and liver (D) from WT (black bars) and CD44 KO (white bars) mice at 24 and 48 hours after infection with 10^4 CFU *K. pneumoniae*. Data are expressed as the means $\pm$ SEM; $n = 8$ mice per group. N.D., not determined. * $P < 0.05$, ** $P < 0.01$ versus WT at the same time point.

dissemination to distant organs, we established the number of *K. pneumoniae* CFU in lung, blood, spleen, and liver harvested from CD44 KO and WT mice at 24 and 48 hours after infection, ie, just before the first deaths occurred. Pulmonary bacterial loads were significantly reduced in CD44 KO mice at 48 hours after infection ($P < 0.05$ versus WT mice), a favorable trend that was already noticeable at 24 hours (Figure 2A). Remarkably, CD44 KO mice displayed a significantly diminished dissemination of the infection, as reflected by lower bacterial loads in blood and spleen at 24 and 48 hours, as well as in liver at 48 hours postinfection (Figure 2, B–D; $P < 0.01$ – 0.05 versus WT mice). Together these results suggest that CD44 deficiency limits bacterial outgrowth and dissemination during severe *Klebsiella* pneumonia, but does not impact on lethality. Of note, we did not measure bacterial loads in mice that had succumbed during the observa-

tional study presented in Figure 1; therefore, although the large differences in bacterial loads in CD44 KO and WT mice at 48 hours after infection and the absence of an effect of CD44 deficiency on lethality suggests that death and bacterial loads are not strictly linked, by design our study is not able to establish such a possible direct relation.

CD44 Deficiency Results in Enhanced Lung Inflammation During Severe Pneumonia

CD44 has been implicated as an important mediator of cell migration and lung inflammation.⁷ We therefore determined the role of CD44 in the induction of pulmonary inflammation in response to *K. pneumoniae* infection. For this lung tissue slides were prepared from CD44 KO and

Figure 3. Prolonged lung inflammation in CD44 KO mice during lethal pneumonia. **A–F:** H&E staining of representative lung tissue slides from WT (**A** and **D**) and CD44 KO (**B** and **E**) mice at 24 (**A–C**) and 48 (**D–F**) hours. **G–I:** Ly6 staining of representative lung tissue slides from WT (**G**) and CD44 KO (**H**) mice 48 hours after intranasal infection with 10^4 CFU *K. pneumoniae*. Lung sections are representative for eight mice per group per time point; original magnification, $\times 10$. Inflammation and Ly6 scores of WT (black bars) and CD44 KO (white bars) mice are expressed as the means $\pm$ SEM ($n = 8$ per group at each time point). ** $P < 0.01$ versus WT.

WT mice 24 and 48 hours after infection and semiquantitatively scored according to the scoring system described in *Materials and Methods*. At 24 hours after infection, both mouse strains already showed extensive edema, interstitial inflammation, pleuritis, bronchitis, and endothelialitis; the extent of lung inflammation did not differ between CD44 KO and WT mice at this time point (Figure 3, A–C; also see Supplemental Table 1 at <http://ajp.amjpathol.org>).^{16,18,19} In contrast, at 48 hours after infection CD44 KO mice displayed relatively enhanced lung inflammation, which was primarily due to more edema, endothelialitis, and bronchitis (Figure 3, D–F; also see Supplemental Table 1 at <http://ajp.amjpathol.org>).^{16,18,19} In addition, at this time point (but not at 24 hours, data not shown) CD44 KO mice showed a higher number of Ly6⁺ neutrophils in lung tissue slides (Figure 3, G–I). CD44 KO and WT mice demonstrated similar cytokine (tumor necrosis factor- α , IL-6, IL-10) and chemokine (keratinocyte-derived cytokine, MIP-2) levels in lung homogenates at both time points (data not shown). Hence, these data suggest that (despite lower bacterial burdens which would be anticipated to result in less lung inflammation) CD44 deficiency is associated with an increased lung inflammatory response after infection with lethal dose *Klebsiella*.

CD44 KO Mice Demonstrate Reduced Expression of IRAK-M, A20, and SOCS3

Recent studies have suggested that CD44 plays a role in the induction of negative regulators of Toll-like receptor (TLR) signaling.^{8,25} Therefore, in light of the enhanced lung inflammation in CD44 KO mice we considered it of interest to study gene expression levels of IRAK-M, A20, and SOCS3 in lung homogenates obtained before, 24 or 48 hours after infection. Of considerable interest, whereas WT mice displayed sustained elevated mRNA levels of these negative TLR regulators, in CD44 KO mice gene expression levels of IRAK-M, A20, and SOCS3 all strongly decreased between 24 and 48 hours (Figure 4, A–C). As a consequence, IRAK-M, A20, and SOCS3 mRNA levels were lower in CD44 KO relative to WT mice at both 24 hours and 48 hours after infection ($P < 0.05$ and $P < 0.01$ for the difference between groups at 24 hours and 48 hours, respectively).

CD44 KO Mice Demonstrate Impaired Resolution of Pulmonary Inflammation During Sublethal *Klebsiella* Pneumonia

In the experiments described above a relatively high infectious dose was used, resulting in lethality from 48 hours onward (Figure 1). Since CD44 has been found to play an important role in the regulation of chronic sterile lung inflammation such as induced by intratracheal administration of bleomycin,¹² we next wished to study the impact of CD44 deficiency on the resolution of lung inflammation after low dose nonlethal respiratory tract infection with *K. pneumoniae*. In a first experiment, we in-

Figure 4. Reduced mRNA expression of negative regulators of Toll-like receptor signaling in lungs from CD44 KO mice. IRAK-M (A), A20 (B), and SOCS-3 (C) mRNA expression in lung homogenates from WT (black bars) and CD44 KO (white bars) mice 24 and 48 hours after infection with 10^4 CFU of *K. pneumoniae*. Gene expression is presented as a ratio of the expression of the housekeeping gene β 2-microglobulin (b2m). Data are expressed as the means $\pm$ SEM; $n = 5$ –8 mice per group. * $P < 0.05$, ** $P < 0.01$ versus WT at the same time point. Dashed lines depict levels of uninfected mice.

fecting CD44 KO and WT mice with 175 CFU *Klebsiella*, seeking to sacrifice the animals 5 days postinfection. Much to our surprise, CD44 KO but not WT mice started to die from 3 days onward. At 5 days postinfection 6 of 10 CD44 KO mice had died versus 1 of 10 WT mice ($P < 0.05$). These data hinted to us that CD44 is important for recovery after sublethal infection with *K. pneumoniae*. Considering the bias introduced by these differential survival curves, we next infected CD44 KO and WT mice with 100 CFU of *Klebsiella* with the aim to sacrifice them after 4 or 10 days. At 4 days after infection ($n = 9$ per group), two CD44 KO compared to none of the WT mice had died (not significant), again hinting to accelerated lethality of CD44 KO mice after sublethal *Klebsiella* infection. *Klebsiella* could still be recovered from the lungs of all mice; bacterial loads were not significantly different in CD44 KO compared to WT mice at this time point ($2.5 \pm$

Figure 5. Enhanced pulmonary inflammation in CD44 KO mice during nonlethal pneumonia. Representative lung histology of WT (**A** and **D**) and CD44 KO (**B** and **E**) mice 4 (**A–C**) and 10 (**D–F**) days after intranasal infection with 10^2 CFU *K. pneumoniae*. Lung sections are representative for 7–9 mice per group per time point. H&E staining, original magnification, $\times 10$. Inflammation scores of WT (black bars) and CD44 KO (white bars) mice are expressed as the means $\pm$ SEM ($n = 7$ –9 per group at each time point). ** $P < 0.01$ versus WT at the same time point.

1.1×10^6 versus $7.6 \pm 3.9 \times 10^6$ CFU/ml lung homogenate, respectively). After 10 days ($n = 10$ per group), three mice from both groups had died and all remaining mice had completely cleared the bacteria. Lung inflammation scores were again significantly higher in CD44 KO mice as compared to WT mice, both at 4 and 10 days after infection (both $P < 0.01$; Figure 5, A–F; also see Supplemental Table 2 at <http://ajp.amjpathol.org>). At 4 days the difference between CD44 KO and WT mice was mainly due to more edema and interstitial inflammation, and to a lesser extent to more endothelialitis and bronchitis. At 10 days, WT lungs demonstrated minimal interstitial inflammation, whereas in CD44 KO mice interstitial inflammation was still prominent with clear endothelialitis, bronchitis, and aggregates of neutrophils and macrophages. The prolonged presence of neutrophils in lungs from CD44 KO mice was further indicated by enhanced myeloperoxidase levels in lung homogenates of these

mice at the latter time point ($P < 0.01$, data not shown). We also performed BAL to obtain insight into leukocyte numbers in the bronchoalveolar space. These analyses revealed increased total leukocyte numbers in BALF of CD44 KO mice at both 4 and 10 days after infection (Table 1); at 4 days this was caused by much higher neutrophil counts, whereas at 10 days especially lymphocyte counts were higher in BALF of CD44 KO mice, although the difference with WT mice did not reach statistical significance due to a relatively large interindividual variation (both $P = 0.05$ versus WT mice); macrophage numbers did not differ between mouse strains at either time point. In addition, 4 days after sublethal *K. pneumoniae* infection, the levels of cytokines (tumor necrosis factor- α , IL-6, and IL-1 β) and chemokines (MIP-2 and LPS-induced CXC chemokine) were significantly higher in lung homogenates of CD44 KO mice than of WT mice, further indicative of enhanced pulmonary inflammation

Table 1. CD44 KO Mice Demonstrate Enhanced Numbers of Leukocytes in the Bronchoalveolar Space During Sublethal *Klebsiella Pneumonia*

BALF	Total leukocytes $\times 10^4$ /ml	Neutrophils $\times 10^4$ /ml	Lymphocytes $\times 10^4$ /ml	Macrophages $\times 10^4$ /ml
4 Days				
WT	49.95 $\pm$ 20.79	45.5 $\pm$ 23.6	0.86 $\pm$ 0.66	10.8 $\pm$ 3.82
CD44 KO	202.2 $\pm$ 68.79	192.1 $\pm$ 67.1	0.09 $\pm$ 0.09	12.0 $\pm$ 3.17
10 Days				
WT	9.202 $\pm$ 0.386	0.14 $\pm$ 0.04	0.36 $\pm$ 0.13	8.70 $\pm$ 0.36
CD44 KO	12.29 $\pm$ 0.657**	0.90 $\pm$ 0.49	2.13 $\pm$ 0.74	9.23 $\pm$ 0.44

Total leukocyte counts, neutrophil, lymphocyte, and macrophage numbers in BALF 4 and 10 days after intranasal infection with *K. pneumoniae* (100 CFU) in WT and CD44 KO mice. Data are expressed as the means $\pm$ SEM of 5–8 mice per group at each time point.
 ** $P < 0.01$ versus WT mice.

Table 2. CD44 KO Mice Demonstrate Elevated Pulmonary Cytokine and Chemokine Concentrations 4 Days After Intranasal Infection with a Sublethal Dose of *K. pneumoniae*

	TNF- α (ng/ml)	IL-6 (ng/ml)	IL-1 β (ng/ml)	KC (ng/ml)	MIP-2 (ng/ml)	LIX (ng/ml)
WT	1.24 $\pm$ 0.14	1.16 $\pm$ 0.28	0.54 $\pm$ 0.12	9.57 $\pm$ 2.93	8.97 $\pm$ 2.03	3.33 $\pm$ 0.34
CD44 KO	3.24 $\pm$ 0.64***	3.05 $\pm$ 0.44**	1.91 $\pm$ 0.55*	17.7 $\pm$ 5.68	29.6 $\pm$ 8.91*	4.69 $\pm$ 0.26**

Proinflammatory cytokine (TNF- α , IL-6, and IL-1 β) and chemokine (KC, MIP-2, and LIX) levels in lung 4 days after intranasal infection with *K. pneumoniae* (100 CFU) in WT and CD44 KO mice. Data are expressed as the means $\pm$ SEM; n = 7–8.

* P < 0.05 versus WT mice.

** P < 0.01 versus WT mice.

*** P < 0.001 versus WT mice.

TNF, tumor necrosis factor.

(Table 2). At 10 days all cytokine levels had decreased as compared to 4 days and were similar in both groups (data not shown). Cytokine and chemokine levels in lung homogenates of uninfected mice were very low and not different between CD44 KO and WT mice (data not shown). As it has been shown that HA levels accumulate in the absence of CD44 during noninfectious lung injury,^{8,12} we measured HA concentrations in BALF. HA concentrations were increased at 4 days after infection as compared to baseline and declined again after 10 days in both WT and CD44 KO mice; however, at both time points HA levels were significantly elevated in BALF of CD44 KO mice relative to HA concentrations in BALF of WT mice (P < 0.01, Figure 6A). As short-fragment HA can induce a proinflammatory response in the pulmonary compartment,^{26–28} we determined HA size in whole lungs from WT and CD44 KO mice. At 4 days after *Klebsiella* infection HA size from CD44 KO was smaller than HA from WT mice (WT peak molecular weight 1097–1557 kDa, CD44 KO 11–481 kDa), whereas at 10 days no significant difference in HA size was found between groups (WT 31–432 kDa, CD44 KO 60–481 kDa) (Figure 6B).

CD44 KO Mice Demonstrate a Prolonged Lung Inflammatory Response on Intrapulmonary Delivery of *Klebsiella* LPS

The experiments using viable *Klebsiella* described above established that CD44 exerts a dual effect on the host response to *K. pneumoniae*: it impacts on the growth and dissemination of bacteria and it influences the resolution of lung inflammation. To eliminate the possible influence of different bacterial loads on lung inflammation, we performed experiments in which CD44 KO and WT mice were intranasally administered with *Klebsiella* LPS and euthanized 1, 2, or 4 days later. At 1 and 2 days, lung inflammation scores did not differ between CD44 KO and WT mice (Figure 7, A–F; also see Supplemental Table 3 at <http://ajp.amjpathol.org>). At 4 days, however, pulmonary inflammation had started to resolve in WT mice, but to a much lesser extent in CD44 KO mice (Figure 7, G–I). At this time point, the difference in inflammation scores was especially due to prolonged presence of endothelialitis and edema in CD44 KO lungs (see Supplemental Table 1 at <http://ajp.amjpathol.org>).^{16,18,19} To obtain insight into the impact of CD44 on leukocyte influx into the bronchoalveolar space on intrapulmonary delivery of *Kleb-*

siella LPS, we performed BAL and determined leukocyte counts and differentials in the fluid harvested (Table 3). At 1 day after LPS administration leukocyte numbers were decreased in CD44 KO as compared to WT mice, which was mainly due to significantly reduced neutrophil

Figure 6. Elevated hyaluronan levels in the bronchoalveolar space of CD44 KO mice during nonlethal pneumonia. **A:** HA concentrations in BALF from WT (closed symbols) and CD44 KO (open symbols) mice before, 4, and 10 days after infection with 10^2 CFU *K. pneumoniae*. Data are expressed as the means $\pm$ SEM. ** P < 0.01 versus WT at the same time point. **B:** Agar gel electrophoresis of total lung HA, visualized with staining with Stains-All. HA samples for the gel were from combined multiple mouse lung tissues in each group (day 4 WT from nine mice, and others were from seven mice each). **Lane 1,** high-molecular-weight HA ladder. **Lane 2,** low-molecular-weight HA ladder. **Lane 3,** WT mice 4 days postinfection. **Lane 4,** CD44 KO mice 4 days postinfection. **Lane 5,** WT mice 10 days postinfection. **Lane 6,** CD44 KO mice 10 days postinfection.

Figure 7. Prolonged pulmonary inflammation in CD44 KO mice in response to intranasal LPS instillation. Representative lung histology of WT (A, D, and G) and CD44 KO (B, E, and H) mice 1 (A–C), 2 (D–F), and 4 (G–I) days after intranasal administration of 1 μ g of *K. pneumonia*-derived LPS. Lung sections are representative for eight mice per group per time point. H&E staining, original magnification $\times 10$. Inflammation scores of WT (black bars) and CD44 KO (white bars) mice are expressed as the means $\pm$ SEM ($n = 8$ per group at each time point). * $P < 0.05$ versus WT at the same time point.

counts. At 2 days lymphocytes started to appear in both groups and neutrophil numbers in BALF from WT mice decreased to neutrophil counts in BALF from CD44 KO mice; at this time point the number of macrophages was lower in BALF from CD44 KO mice when compared to WT mice. At 4 days after LPS neutrophil numbers decreased as compared to 2 days; however, CD44 KO mice displayed increased numbers of neutrophils and lymphocytes in their BALF as compared to WT mice, whereas macrophage counts did not differ between groups. These data suggest that the resolution of neutrophils is impaired in the absence of CD44 which is accompanied by enhanced lymphocyte recruitment. In addition, 4 days (but not 1 or 2 days; data not shown) after LPS administration, the levels of tumor necrosis factor- α , IL-6, and MIP-2 were significantly higher in lung homogenates of CD44 KO mice as compared to WT mice, further indicative of enhanced inflammation (Table 4). Finally, we measured HA concentrations in BALF; in WT mice HA levels

at 1 day after LPS were increased as compared to baseline and declined again already at 2 days. In contrast, HA levels in CD44 KO BALF did not decline up to 4 days and were significantly enhanced as compared to WT mice ($P < 0.001$ for all time points; Figure 8).

Treatment with HABP Does Not Attenuate the Enhanced Pulmonary Inflammation in CD44 KO Mice with Klebsiella Pneumonia

Low-molecular weight HA has been reported to be responsible, at least in part, for the perpetuation of pulmonary inflammation in models of sterile lung injury.^{20,21} To determine the possible role of low-molecular-weight HA in the enhanced pulmonary inflammation in CD44 KO mice during *Klebsiella pneumonia*, we administered mice with HABP, which has previously been shown to attenuate the lung inflammation associated with intratracheal

Table 3. CD44 KO Mice Show Reduced Recruitment but Prolonged Presence of Neutrophils in the Bronchoalveolar Space After Intranasal Administration of *Klebsiella* LPS

BALF	Total leukocytes $\times 10^4/\text{ml}$	Neutrophils $\times 10^4/\text{ml}$	Lymphocytes $\times 10^4/\text{ml}$	Macrophages $\times 10^4/\text{ml}$
1 Day				
WT	80.69 $\pm$ 10.6	75.33 $\pm$ 10.42	Not present	5.38 $\pm$ 0.80
CD44 KO	38.19 $\pm$ 4.82*	34.55 $\pm$ 5.15*	Not present	3.65 $\pm$ 0.61
2 Days				
WT	36.62 $\pm$ 5.15	22.91 $\pm$ 4.76	0.79 $\pm$ 0.51	12.9 $\pm$ 1.68
CD44 KO	31.37 $\pm$ 4.91	22.33 $\pm$ 5.37	1.73 $\pm$ 0.68	7.30 $\pm$ 0.93*
4 Days				
WT	19.42 $\pm$ 2.05	0.93 $\pm$ 0.19	1.70 $\pm$ 0.28	16.8 $\pm$ 1.77
CD44 KO	33.90 $\pm$ 4.14*	1.65 $\pm$ 0.28*	14.3 $\pm$ 2.74**	17.8 $\pm$ 1.56

Total leukocyte counts, neutrophil, lymphocyte, and macrophage numbers in BALF at 1, 2, and 4 days after intranasal administration of *Klebsiella* LPS (1 μg) in WT and CD44 KO mice. Data are expressed as the means $\pm$ SEM; $n = 5-8$.

* $P < 0.05$ versus WT mice.

** $P < 0.01$ versus WT mice.

bleomycin administration and ozone inhalation.^{20,21} Four days after infection with 100 *Klebsiella* CFU CD44 KO mice treated with a scrambled control peptide displayed increased pulmonary inflammation when compared to WT mice treated with this peptide ($P < 0.001$, Figure 9), confirming the data shown in Figure 3, D–F. Importantly, treatment of CD44 KO mice with HABP did not reduce pulmonary inflammation as compared to scrambled control treatment.

Discussion

To the best of our knowledge only one previous investigation studied the role of CD44 in host defense against bacterial pneumonia, reporting enhanced neutrophil accumulation and increased pulmonary edema 6 hours after intrapulmonary delivery of *E. coli*.⁹ In that study CD44 deficiency did not impact on the early host response to *S. pneumoniae* in the lung, while the effect of CD44 deficiency on the immune response beyond the first 6 hours was not addressed in experimentally induced airway infection by either pathogen.⁹ We argued that CD44 may affect the course of bacterial pneumonia in several stages, considering its role as a regulator of cytokine production in and leukocyte migration to the lung^{7–9} and considering its pivotal function in the clearance of inflammation in models of sterile lung injury.^{8,12} Therefore, in the present study we sought to investigate the functional role of CD44 during both the induction and resolution phase of inflammation during *Klebsiella* pneumonia. We here demonstrate that during lethal pneumonia CD44 deficiency limits bacterial outgrowth and dissemination while concurrently enhancing lung pathology.

We confirmed an important role for CD44 during sublethal pneumonia, revealing an enhanced resolution of lung inflammation and a reduced lethality in the presence of CD44. Our data identify CD44 as a vital regulator of lung inflammation during various stages of *Klebsiella* pneumonia.

To obtain insight into the role of CD44 in host defense against *K. pneumoniae*, we first used a bacterial dose that causes pneumonia and sepsis, and lethality in normal immunocompetent mice.^{16,18} CD44 did not impact on lethality during severe *Klebsiella* pneumonia. Whereas pulmonary bacterial loads were similar at 24 hours, at 48 hours after infection bacterial outgrowth was reduced in lungs of CD44 KO mice. Strikingly, dissemination of *K. pneumoniae* to distant body compartments was diminished already after 24 hours of infection, and even more so after 48 hours. Of note, CD44 has been described to mediate phagocytosis of bacteria such as *M. tuberculosis*¹⁴ and *S. aureus*.¹³ However, in preliminary experiments we could not identify such a role for CD44 on neutrophils or macrophages in phagocytosis of *K. pneumoniae* (data not shown). Possibly, the influence of CD44 on the growth and spreading of *Klebsiella* after infection with a lethal dose is secondary to its impact on the local inflammatory response. Indeed, CD44 deficiency resulted in enhanced lung inflammation at 48 hours, which may benefit local antibacterial effector mechanisms exerted by the innate immune system at the primary site of infection.^{29,30}

CD44 deficiency consistently resulted in enhanced pulmonary inflammation during *Klebsiella* pneumonia as determined by semiquantitative scoring of specific components of the inflammatory response in the lung. In lethal

Table 4. CD44 KO Mice Demonstrate Elevated Pulmonary Cytokine and Chemokine Concentrations 4 Days After Intranasal Inoculation with *Klebsiella* LPS

	TNF- α (ng/ml)	IL-6 (ng/ml)	IL-1 β (ng/ml)	KC (ng/ml)	MIP-2 (ng/ml)
WT	1.88 $\pm$ 0.09	0.08 $\pm$ 0.004	0.079 $\pm$ 0.006	0.35 $\pm$ 0.04	0.54 $\pm$ 0.02
CD44 KO	2.68 $\pm$ 0.23*	0.11 $\pm$ 0.010*	0.127 $\pm$ 0.027	0.97 $\pm$ 0.43	0.83 $\pm$ 0.08**

Proinflammatory cytokine (TNF- α , IL-6, and IL-1 β) and chemokine (KC and MIP-2) levels in lung homogenates obtained from WT and CD44 KO mice 4 days after intranasal administration of *Klebsiella* LPS (1 μg). Data are expressed as the means $\pm$ SEM; $n = 8$.

* $P < 0.05$ versus WT mice.

** $P < 0.01$ versus WT mice.

Figure 8. Elevated hyaluronan levels in the bronchoalveolar space of CD44 KO mice in response to intranasal LPS instillation. HA concentrations in BALF from WT (closed symbols) and CD44 KO (open symbols) mice before, 1, 2, and 4 days after intranasal administration of LPS. Data are expressed as the means $\pm$ SEM. *** $P < 0.001$ versus WT at the same time point.

pneumonia the impact of CD44 deficiency on lung pathology likely was underestimated in our experiments, considering that the reduced bacterial loads in lungs of CD44 KO mice would be anticipated to result in less lung inflammation. Conceivably, the increased lung pathology in CD44 KO mice adversely affected the potentially beneficial effect of reduced bacterial growth and dissemination in these mice, thereby explaining the lack of impact of CD44 on survival during lethal *Klebsiella* pneumonia. Alternatively, the challenge administered in these high-dose experiments was too severe to visualize a potential protective effect of CD44. In accordance with this notion, CD44 KO mice showed mortality after infection with a lower *Klebsiella* dose that did not cause lethality in WT mice. Notably, CD44 KO mice demonstrated enhanced interstitial lung edema and inflammation in these low-dose experiments; it remains to be established whether this negatively impacted on gas exchange in the lung and thereby on survival.

We found increased neutrophil numbers in lungs from CD44 KO mice during both lethal pneumonia and the resolution phase of sublethal pneumonia, as well as 4 days after intrapulmonary delivery of *Klebsiella* LPS. In accordance, *in vitro* experiments demonstrated that CD44 negatively regulates epithelium-neutrophil interactions and thereby migration across an epithelial layer.³¹ Moreover, migration of CD44 KO neutrophils through Matrigel toward a gradient of fMLP, a bacteria-derived

molecule that is a potent chemoattractant for neutrophils during pneumonia,³² was enhanced when compared to migration of WT neutrophils.⁹ Of note, in our *Klebsiella* LPS experiments we found fewer neutrophils in BALF from CD44 KO as compared to WT mice at 1 day after the challenge. In line with this result, aerosol administration of a low dose of *E. coli*-derived LPS resulted in reduced neutrophil recruitment to the lungs of CD44 KO mice,³³ and a positive role of CD44 in neutrophil recruitment has also been shown in other models like allergic dermatitis,³⁴ renal ischemia reperfusion,¹⁰ and liver inflammation.^{35,36} As such, the role of CD44 in neutrophil trafficking seems to depend on the timing, type, and severity of the inflammatory stimulus. We found no differences in macrophage numbers during *Klebsiella* pneumonia, indicating that CD44 deficiency does not impact on macrophage recruitment into the bronchoalveolar space induced by *K. pneumoniae*. Of note, at 2 days after LPS macrophages were less abundant in BALF from CD44 KO mice than from WT mice. The role of CD44 in macrophage trafficking toward the lung is complex; in models of sterile lung inflammation elicited by intrapulmonary delivery of either LPS or bleomycin CD44 KO mice demonstrated increased macrophage numbers in their BALF,^{8,12} whereas decreased pulmonary macrophage numbers were found in CD44 KO mice after aerosol challenge with a low dose of LPS,³³ during ozone-induced airway hyperresponsiveness²⁰ and tuberculosis.¹⁴ Moreover, we observed increased lymphocyte numbers in lungs from CD44 KO mice during the resolution phase of both sublethal pneumonia and LPS. CD44 has been shown to be required for extravasation of activated lymphocytes into the peritoneal cavity.^{37,38} As we found similar lymphocyte numbers in BALF from WT and CD44 KO mice in the acute inflammatory phase, the enhanced lymphocyte counts in a later stage likely are a result of prolonged presence or delayed recruitment rather than enhanced early recruitment.

CD44 may attenuate lung inflammation via several mutually nonexclusive mechanisms. CD44 may inhibit the release of proinflammatory cytokines by macrophages on stimulation by TLR agonists.^{8,39} In addition, CD44 has been reported to positively regulate the induction of negative regulators of TLR signaling.^{8,25} We found reduced gene expression levels of IRAK-M, A20, and SOCS3 in lung homogenates of CD44 KO mice at 24 and 48 hours, which may have contributed to the enhanced pulmonary inflammation at the latter time point. Moreover, CD44 is known to bind and to internalize HA fragments.^{5,40} It has become clear that HA fragments induce nuclear factor κ B activation,⁴¹ and thereby proinflammatory gene expression through TLR2 and TLR4 signaling.^{27,42–44} Indeed, CD44 KO mice showed impaired clearance of HA in bleomycin or *E. coli* LPS induced lung inflammation accompanied by sustained accumulation of inflammatory cells.^{8,12} In line, we found significantly increased HA concentrations in BALF from CD44 KO mice at 4 and 10 days after sublethal pneumonia and from 1 to 4 days after intrapulmonary delivery of *Klebsiella* LPS, suggesting that CD44 facilitates the resolution of inflammation during *Klebsiella* pneumonia at least in part through clearance of

Figure 9. Binding of hyaluronan does not reduce pulmonary inflammation in CD44 KO mice. Inflammation scores of WT (black bars) and CD44 KO (white bars) mice treated with scrambled peptide (scr) and CD44 KO mice treated with HABP (gray bars) 4 days after intranasal infection with 10^2 CFU of *K. pneumoniae*. Data are expressed as the means $\pm$ SEM. *** $P < 0.001$ versus WT.

HA from the lungs. In accordance with earlier findings in models of sterile pulmonary inflammation,^{12,20} we found more low-molecular-weight HA fragments in the lungs of infected CD44 KO mice than in lungs of WT mice (which primarily contained high-molecular-weight HA fragments). Subcutaneous treatment with HABP has previously been shown to attenuate lung inflammation induced by intratracheal bleomycin instillation or ozone inhalation.^{20,21} Further support for a role of HA in local inflammation is derived from studies in which HABP inhibited inflammation during skin wound repair.⁴⁵ However, we here demonstrate that HABP did not inhibit the enhanced lung inflammation in CD44 KO mice during *Klebsiella* pneumonia. In addition, another HABP (Pep-1) has been shown to suppress carrageenan-induced cutaneous inflammation,⁴⁶ and intraperitoneal administration of Pep-1 at a dose previously shown to attenuate lung inflammation associated with bleomycin²⁷ did not impact on the increased pulmonary inflammation in CD44 KO mice either (data not shown). Together these data suggest that accumulation of proinflammatory low-molecular-weight HA fragments does not contribute to the perpetuation of lung inflammation during *Klebsiella* pneumonia in CD44 KO mice to a significant extent.

The most consistent finding in the different models used (lethal infection, nonlethal infection, LPS administration) increased lung inflammation in CD44 KO mice, as determined by semiquantitative pathology scores of lung tissue slides. Other host response parameters were not consistently affected in CD44 KO mice in different experimental approaches, which may have been caused by the infectious dose chosen and as a consequence thereof the severity of the model. This could possibly explain why the adverse effect of CD44 deficiency on survival was only revealed after low-dose infection (in the lethal model, wherein virtually all WT mice died, such an adverse effect is not easily demonstrated). The fact that CD44 deficiency was associated with increased pulmonary cytokine and chemokine levels after low-dose infection and LPS administration, but not after lethal infection, may have been caused by the lower bacterial loads in CD44 KO mice in the lethal model, providing a less potent proinflammatory stimulus, thereby masking the effect of CD44 deficiency on cytokine production. Finally, the fact that CD44 deficiency resulted in lower bacterial growth and dissemination after lethal infection, but not after low dose infection, could be explained by the fact that a beneficial effect on antibacterial defense is not easily shown in a model in which virtually all WT mice clear the bacteria. Hence, although our study is limited in that it does not expose direct mechanistic links between bacterial growth, lung inflammation, and mortality, the combined data suggest that CD44 deficiency renders mice more vulnerable to lethality after low-dose infection at least in part by exaggerating lung pathology. Together these results reveal CD44 as a key controller in various stages of the host response to Gram-negative pneumonia.

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