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Chapter 2

CD44 is protective during hyperoxia induced lung injury

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Submitted
Abstract

Patients with acute lung injury or respiratory distress syndrome often require supplemental oxygen to maintain tissue oxygenation. Although this supportive treatment is necessary, it can also cause or worsen lung inflammation. CD44 is a transmembrane adhesion molecule that is present on a wide variety of cell types, including leukocytes and parenchymal cells, and an important player in leukocyte trafficking. The aim of this study was to determine the role of CD44 during hyperoxia induced acute lung injury. CD44 knockout (KO) and wild-type (WT) mice were exposed to either > 95% oxygen or room air for 24-72 hours. Whereas all WT mice survived the 72-hour observation period, 37.5% of CD44 KO mice died. CD44 deficiency was associated with a profound influx of neutrophils into the bronchoalveolar space, in the presence of similar or even lower neutrophil numbers in lung parenchyma, suggesting that CD44 is important for containing neutrophils in the pulmonary interstitium during hyperoxia. In addition, CD44 deficiency resulted in enhanced interleukin-6 and keratinocyte-derived chemokine release into bronchoalveolar lavage fluid (BALF). CD44 KO mice further displayed evidence for increased vascular leak (reflected by higher protein levels in BALF) and injury of type II respiratory epithelial cells (higher BALF alkaline phosphatase levels). Strikingly, CD44 protected against bronchial epithelial cell death, as shown by enhanced epithelial cell necrosis and increased BALF nucleosome levels in CD44 KO mice. Osteopontin, an important ligand for CD44, was constitutively expressed in BALF of naive mice, increasing after 72 hours of hyperoxia. However, osteopontin KO mice were indistinguishable from WT mice during exposure to hyperoxia for up to 72 hours. These data suggest that CD44 protects against hyperoxia induced mortality and lung injury by a mechanism that does not rely on its interaction with osteopontin.
Introduction

In the past years advances have been made in the mechanical ventilation of patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) (1, 2). Patients with ALI/ARDS almost invariably are given supplemental oxygen to maintain tissue oxygenation. However, prolonged exposure to high oxygen concentrations can worsen or induce lung damage in already injured or previously healthy lungs (3). The pathogenesis of hyperoxia induced lung injury is not completely understood and insight into factors involved could help to identify possible new targets for improving the management of patients who require supplemental oxygen. The inflammatory response to hyperoxia is dominated by the recruitment of neutrophils into the bronchoalveolar space (1, 3). The influx of neutrophils into the lungs upon exposure to hyperoxia likely is mediated in part by the local production of CXC chemokines and the expression of CXCR2, the main neutrophil receptor for CXC chemokines in rodents (4-6). In addition, increased expression of adhesion molecules on the pulmonary endothelial cell surface has been found to contribute to neutrophil trafficking into the bronchoalveolar compartment during hyperoxia (7).

CD44 is a transmembrane adhesion molecule that is present on a wide variety of cell types, including leukocytes and parenchymal cells (8, 9). Important ligands for CD44 include hyaluronic acid (HA) (10) and osteopontin (OPN) (11). Different roles for CD44 in neutrophil and macrophage (trans)migration have been described in vitro and in vivo (12-17). For example, during ozone-induced airway hyperresponsiveness inflammatory cell influx into the lungs was dependent on HA and CD44, and thus impaired in the absence of CD44 (18). On the other hand, in a model of acute pulmonary lung inflammation induced by intratracheal lipopolysaccharide (LPS) administration, neutrophils appeared earlier in bronchoalveolar lavage fluid (BALF) of CD44 deficient than in BALF of wild-type (WT) mice (12). At present it is unknown whether CD44 is involved in hyperoxia-induced neutrophil migration and lung injury. Therefore, in the present study we sought to investigate the function of CD44 in the induction of lung inflammation upon exposure to hyperoxia.
Materials & Methods

Animals
Male 10-12 week old WT C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). CD44 knock-out (KO) mice (kindly provided by Dr. A. Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands (19)) and OPN KO mice (Jackson Laboratories, Bar Harbor, ME), both on a C57BL/6 genetic background, were bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Exposure to hyperoxia
Mice were exposed to either > 95% oxygen or room air under normobaric pressure in a 90 x 70 x 70 cm chamber. At time points indicated, mice were anesthetized with ketamine (Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (Pfizer Animal Health BV, Capelle aan de IJssel, The Netherlands) for collection of the samples as described below.

Bronchoalveolar lavage
BALF was obtained and differential counts were carried out as described earlier (20, 21). Briefly, the trachea was exposed through a midline incision and BALF was harvested by instilling and retrieving 1 mL (in aliquots) of sterile phosphate buffered saline. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA) and differential cell counts were performed on cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). To obtain lung homogenates, lungs were removed aseptically, and homogenized in 5 volumes of sterile 0.9% NaCl using a tissue homogenizer (Biospec Products, Bartlesville, OK).

Assays
Myeloperoxidase (MPO), keratinocyte-derived chemokine (KC), interleukin (IL)-6, soluble receptor for advanced glycation end products (RAGE) and OPN levels were determined by ELISA (MPO; Hycult, Uden, the Netherlands, others; R&D Systems, Abingdon, United Kingdom). Protein levels were measured using the bicinchoninic acid protein kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Alkaline phosphatase levels were determined by a commercially available kit (Sigma...
Aldrich, St. Louis, MO) using a Hitachi analyzer (Roche, Mannheim, Germany). Nucleosome levels were determined by means of ELISA as described previously (22) with some modifications. In brief, ELISA plates were coated with monoclonal anti-histone H3 antibody (CLB/ANA-60) and the samples were added and incubated for 1 h at room temperature. After washing, biotin-labelled F(ab')2 fragments of monoclonal antinucleosome antibody (CLB/ANA-58) were added and incubated for another hour at room temperature. Binding of biotin-labelled antibodies was detected with streptavidin-horse radish peroxidase (HRP) using tetramethylbenzidine (TMB) as a substrate. The reaction was stopped with 2 M H₂SO₄ and the absorbance was measured at 450 nm. Serial dilutions of a culture supernatant of approximately 1 × 10⁶/ml Jurkat cells, cultured for a week with etoposide to obtain 100% dead cells were used as a standard. BALF levels were expressed as units/milliliter. One unit was arbitrarily set at the amount of nucleosomes released by approximately 100 Jurkat cells. The detection limit of the ELISA was 0.8 U/ml.

**Histology**

Lungs were fixed in 10% formalin and embedded in paraffin. 4µm thick sections were stained with hematoxylin-eosin or periodic acid Schiff after diastase (PAS-D). The following parameters were scored: interstitial inflammation, endothelialitis, edema, and pleuritis. Each parameter was graded on a scale of 0 to 4 (0, absent; 1, mild; 2, moderate; severe; and 4, very severe). The total pathology score was expressed as the sum of the score for all parameters. Necrosis of the bronchial epithelium was scored on PAS-D stained tissues (on a similar scale of 0 to 4). Granulocyte staining was performed using fluorescein isothiocyanate-labeled anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA) as described earlier (23, 24). 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. Coloured areas were analysed with Image Pro Plus (Media Cybernetics, Bethesda, MD) and expressed as percentage of the total surface area. The average of ten pictures was used for analysis.

**Statistical analysis**

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests and survival was compared by log-rank test, using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). Values of P < 0.05 were considered statistically significant.
Results

**CD44 protects against hyperoxia induced mortality**

To obtain insight in the role of CD44 in hyperoxia induced lung injury CD44 KO and WT mice were exposed to > 95% oxygen for up to 72 h; control mice were exposed to room air. Whereas all 16 WT mice remained alive during this observation period, 6/16 CD44 KO mice died, with the first deaths occurring after 48 h ($P = 0.007$, Figure 1). Samples for further measurements were obtained after 24, 48 or 72 h of hyperoxia; please note that these data are somewhat biased in favour of WT mice since (the most severely ill) CD44 KO mice started dying from 48 h onward.

**CD44 prevents neutrophil recruitment to the bronchoalveolar space during hyperoxia**

The inflammatory response to hyperoxia is dominated by the recruitment of neutrophils into the bronchoalveolar space (1, 3). CD44 KO mice displayed a profound influx of neutrophils into BALF, which became apparent after 24 h and increased further up to the end of the 72 h observation period. Within this time frame, WT mice demonstrated a very modest influx of neutrophils ($P < 0.05$ and $P < 0.01$ versus WT for $t=48$ and 72 h respectively, Figure 2A). To examine whether the increased influx of CD44 KO neutrophils into the bronchoalveolar space was accompanied with a concurrent accumulation of neutrophils in lung tissue, we determined the number of neutrophils in pulmonary interstitium by means of Ly-6 staining of histopathology slides and MPO measurements in lung homogenates (Figure 2B and C). Remarkably and unlike the rapid and strong recruitment of neutrophils into BALF of CD44 KO
mice after 24 h of hyperoxia the number of Ly6+ cells in lung tissue and lung MPO levels were similar in CD44 KO and WT mice; at 48 h lungs of CD44 KO mice even contained significantly fewer neutrophils, as reflected by both fewer Ly6+ cells in lung tissue slides and lower pulmonary MPO concentrations (both \( P < 0.05 \) versus WT mice). At 72 h of hyperoxia pulmonary neutrophil numbers were further increased and similar in both groups. Taken together, these data suggest that during hyperoxia CD44 inhibits the transition of neutrophils to the bronchoalveolar space.

**Figure 2. CD44 prevents neutrophil recruitment to the bronchoalveolar space during hyperoxia.** Neutrophil numbers in (A) BALF, and in (B-C) lung tissue as determined by Ly-6 staining of lung tissue slides (B) and myeloperoxidase (MPO) measurement in lung homogenates by ELISA (C) in WT (black bars) and CD44 KO mice (white bars) exposed to > 95% oxygen and sacrificed after 24, 48 or 72 h. Data are means ± SEM, n = 6-8/group/time point. * \( P < 0.05 \), ** \( P < 0.01 \) as compared to WT mice.

**CD44 KO mice demonstrate enhanced KC and IL-6 concentrations in BALF**
Chemokines and cytokines play an important role during hyperoxia induced lung injury (6, 7). To further evaluate the pulmonary responses to oxygen exposure, we measured IL-6 and KC in BALF (Figure 3). After 24 h of hyperoxia IL-6 and KC were not detectable yet. However, at 48 h both mediators were enhanced in CD44 KO mice (\( P < 0.01 \) and \( P = 0.05 \) versus WT at 48 h, respectively) and further induced at 72 h, when the levels in CD44 KO mice were approximately 4 and 10 times higher for IL-6 and KC respectively as compared to WT mice (\( P < 0.01 \) for both mediators).

**CD44 KO mice show more severe lung injury during hyperoxia**
Having shown that CD44 KO mice displayed an increased migration of neutrophils to the bronchoalveolar space, we next evaluated the role of CD44 in lung inflammation and damage during hyperoxia. Therefore, we analyzed lung tissue slides from WT and CD44 KO mice exposed to hyperoxia for 24, 48 or 72 h. Upon histological examination, the lungs of hyperoxia exposed WT and CD44 KO mice showed interstitial...
inflammation together with edema and pleuritis in most animals already after 24 h of hyperoxia, which was not further increased at 72 h (Figure 4A-I). The mean pathology score of lungs using the scoring system described in the Methods section was similar in both groups at all time points. Strikingly, after 48 h of hyperoxia necrosis of the bronchial epithelium was more prominent in CD44 KO than in WT mice (Figure 5). At 72 h determination of bronchial epithelial necrosis was not possible anymore because of extreme degradation of the epithelial layer in both mouse strains (data not shown). The extent of lung injury was further determined by measuring total protein and alkaline phosphatase in BALF (25, 26). Total protein levels at 24 h after hyperoxia remained at the level of room air exposed mice, while after 48 h protein levels started to increase in both mouse strains, to become profoundly elevated at 72 h after hyperoxia. At this time point protein levels were significantly higher in CD44 KO mice as compared to WT mice ($P < 0.05$, Figure 6A). Alkaline phosphatase was detectable only after 72 h, when significantly increased levels were found in CD44 KO as compared to WT mice ($P < 0.01$, Figure 6B). sRAGE has recently been described as a marker of lung injury based on experimental studies in mice during hyperoxia (25), and in rats and in patients with ALI (27). Indeed, sRAGE levels strongly increased during hyperoxia as compared to room air; sRAGE levels were similar in WT and CD44 KO mice (Figure 6C). Finally, we measured nucleosomes as a marker for apoptotic cell death (28). After 24 and 48 h of hyperoxia nucleosome levels were below the detection limit. However, at 72 h nucleosomes were detectable in BALF of both groups, with higher levels in CD44 KO mice (Figure 6D, $P = 0.08$ versus WT mice).
Figure 4: CD44 deficiency does not impact on the extent of lung pathology. Representative HE stainings of lung tissue of WT (A, D and G) and CD44 KO (B, E and H) mice exposed to > 95% oxygen for 24 (A-C), 48 (D-F) and 72 h (G-I). Original microscopic magnification: 20x. Inflammation scores are expressed as means ± SEM (WT mice: black bars; CD44 KO mice: white bars, n = 6-8/group/time point).

Together, these data suggest that CD44 protects against epithelial cell death and lung injury during hyperoxia.

Osteopontin does not impact on hyperoxia induced lung injury

OPN is an important ligand of CD44 (11). To determine whether OPN could be involved in the protective role of CD44 during hyperoxia, we first measured OPN concentrations in BALF of WT and CD44 KO mice. OPN levels in BALF from mice exposed to hyperoxia did not increase as compared to BALF of mice exposed to room air levels up to 48 h (data not shown). At 72 h BALF OPN had increased in both mouse groups, but to a larger extent in CD44 KO mice (Figure 7A, P = 0.05 versus WT mice). To determine a possible role for endogenous OPN in hyperoxia induced lung injury, we compared the pulmonary response to hyperoxia in OPN KO and WT mice after 48 and 72 h of exposure, following a similar approach as described above for CD44 KO mice.
Figure 5: CD44 deficiency exaggerates epithelial cell necrosis. Representative PAS-D stainings of lung tissue of WT (A) and CD44 KO (B) mice exposed to > 95% oxygen for 48 h. Original microscopic magnification: 40x. Graphical representation of the degree of necrosis of bronchial epithelium (C) determined according to the scoring system described in the Methods section. More profound necrosis of the bronchial epithelium was observed in the CD44 KO mice compared to WT mice. Data are means ± SEM of 8 mice per group. ** P < 0.01 as compared to WT mice.

OPN KO and WT mice did not differ with respect to mortality, neutrophil recruitment into BALF, the concentrations of IL-6, KC, total protein, alkaline phosphatase or nucleosomes in BALF or histopathology of the lung at either 48 or 72 h of hyperoxia.

Figure 6: Enhanced total protein and alkaline phosphatase levels in CD44 KO mice. WT (black bars) and CD44 KO mice (white bars) were exposed to > 95% oxygen or room air and sacrificed after 24, 48 or 72 h. Total protein (A), alkaline phosphatase (AP) (B), sRAGE (C) and nucleosomes (D) were measured in BALF. Alkaline phosphatase and nucleosomes were only detectable at 72 h of hyperoxia. Data are means ± SEM of 5-8/group/time point. b.d. means below detection limit. * P < 0.05, ** P < 0.01 as compared to WT mice.
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(shown for IL-6, KC, total protein, and alkaline phosphatase at 72 h in Figure 7B-E). Together, these data suggest that OPN does not contribute to the protective role of CD44 during hyperoxia.

Figure 7: Osteopontin does not contribute to hyperoxia induced lung injury. WT (black bars) and CD44 KO (white bars) mice (A) or WT and osteopontin (OPN) KO mice (grey bars) (B-E) were exposed to > 95% oxygen and sacrificed after 72 h. OPN (A), IL-6 (B), KC (C), total protein (D) and alkaline phosphatase (AP) (E) were measured in BALF. Data are means ± SEM of 5-8 mice per group. Dashed lines represent room air levels.

Discussion

CD44 is abundantly expressed and plays a role in several inflammatory processes, including inflammatory cell recruitment and cytokine and chemokine responses (12, 14, 15, 29-34). We here demonstrate that CD44 protects against hyperoxia induced lung injury and mortality. CD44 especially appeared to protect the respiratory epithelium against hyperoxia induced damage, as indicated by increased epithelial necrosis in CD44 KO mice accompanied by elevated levels of epithelial cell injury markers in BALF. Moreover, we show that an interaction between CD44 and one of its main ligands OPN does not contribute to the protective function of CD44 during hyperoxia.

The recruitment of neutrophils into the bronchoalveolar space dominates the inflammatory response to hyperoxia (1, 3). CD44 on murine neutrophils has been shown to be a ligand for E-selectin (35) and to be important for polarization and
directed migration of these cells (16). Based on these data one might expect that neutrophil recruitment would be impaired in the absence of CD44. However, we found that neutrophils hardly appeared in the bronchoalveolar space of WT mice within our observation period, whereas in CD44 KO mice neutrophil influx was detected from 24 h onward, increasing up to 72 h of hyperoxia. Similarly, enhanced neutrophil recruitment in the absence of CD44 has been reported in several inflammation and infection models including zymosan induced arthritis (31), intratracheal LPS administration (12), *Escherichia coli* induced pneumonia (14), and *M. tuberculosis* infection (34). On the other hand, we and others showed impaired (17, 36, 37) or unaltered (see Chapter 3) neutrophil recruitment during inflammation in the absence of CD44. It has been suggested that the need for CD44 in cellular diapedesis from the circulation depends on vascular integrity and shear stress, and thus on the severity of tissue damage and the organ involved (18). As such, the increased neutrophil influx into BALF of CD44 KO mice during hyperoxia might be explained by the fact that hyperoxia induced breakdown of vascular integrity renders cell adhesion processes redundant for cellular diapedesis. Of note, after 24 h of hyperoxia CD44 KO and WT mice displayed similar neutrophil numbers in lung parenchyma (as indicated by the number of Ly6+ cells in tissue slides and the concentrations of MPO in lung homogenates), while only CD44 KO mice had neutrophils in their BALF; moreover, at 48 h of hyperoxia CD44 KO mice even demonstrated fewer neutrophils in lung parenchyma and much higher neutrophil numbers in their BALF when compared to WT mice. Together these data suggest that during hyperoxia CD44 is not involved in neutrophil diapedesis from the circulation to the parenchyma and that CD44 impairs the transition of neutrophils from parenchyma to the bronchoalveolar space, resulting in containment of these cells in the pulmonary interstitium in WT mice and transition to BALF in CD44 KO mice. This latter response likely was further facilitated by the elevated BALF levels of KC (4-6) (and possibly IL-6 (24)) in CD44 KO mice.

Although neutrophils were abundantly present in the alveolar compartment of CD44 KO but not of WT mice, we found no differences in the extent of lung pathology, as determined by a semi-quantitative scoring method. Although several investigations have indicated that neutrophils can contribute to hyperoxia induced lung injury, it has become clear that these cells are not absolutely required. Indeed, cyclophosphamide induced neutropenia did not alter the increased lung permeability associated with hyperoxia in rats (38), whereas neutrophil depletion using nitrogen mustard only partially reduced lung edema in rabbits (39). More recently, it has been
shown that antibody-mediated neutrophil depletion does not prevent lung edema or protein leak during hyperoxia in mice, indicating dissociation between neutrophil infiltration and lung injury (7). Hyperoxia induces cell death of pulmonary epithelial cells displaying characteristics of both necrosis and apoptosis (40, 41). Strikingly, we found more prominent necrosis of the bronchial epithelium of CD44 KO mice after 48 h of hyperoxia. Furthermore, CD44 KO mice demonstrated enhanced nucleosome levels at 72 h as compared to WT mice, although this difference did not reach statistical significance possibly due to a bias in favour of WT mice since the most severely ill CD44 KO mice had died at this time point. These results are in line with reports that CD44 stimulation down-regulates Fas-mediated apoptosis of lung cancer cells (42) and that CD44 promotes resistance to apoptosis in murine colonic epithelium (43). The CD44 mediated protective effect on the respiratory epithelium may also at least in part explain why CD44 KO mice demonstrated evidence for increased vascular leak, as reflected by higher protein levels in BALF. As alkaline phosphatase and sRAGE release into the bronchoalveolar space are indicative of type II and type I alveolar epithelial cell injury respectively (26, 27), our data on enhanced BALF alkaline phosphatase levels in CD44 KO mice, but similar levels of sRAGE indicate that the presence of CD44 impacted more on type II than on type I cells.

OPN is an important ligand of CD44 (11). As OPN acts as a chemo-attractant for neutrophils (see chapter 7) (44), we checked whether BALF OPN levels were increased during hyperoxia and, if so, different between CD44 KO and WT mice. OPN was constitutively expressed in BALF of naive mice, only to increase after 72 h of hyperoxia. Although BALF OPN levels were slightly increased in CD44 KO mice at this time point, we established that OPN does not contribute to hyperoxia induced lung injury: all parameters for lung inflammation and/or injury were indistinguishable between OPN KO and WT mice. In preliminary experiments we found elevated BALF levels of HA, another main ligand for CD44, in CD44 KO mice (data not shown), which is in line with the function of CD44 to take up and degrade HA (8), and with several other sterile pulmonary inflammation models, such as induced by bleomycin (32), ozone (18), or LPS (see also Chapter 5) (12). Since HA can exert proinflammatory effects in the lung by mediating inflammatory cell recruitment, cytokine release and apoptosis (45), it is possible that elevated HA levels in BALF of CD44 KO mice contributed to the pulmonary damage in these animals. Further studies are warranted to explore the role of HA in hyperoxia induced lung injury.
In conclusion, our data demonstrate that CD44 protects against hyperoxia induced mortality and lung injury, characterized by reduced neutrophil recruitment, cytokine and chemokine release, and respiratory epithelial cell death.

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