Syndecan-4 Regulates Early Neutrophil Migration and Pulmonary Inflammation in Response to Lipopolysaccharide

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Proteoglycans (PGs) and their associated glycosaminoglycan side chains are effectors of inflammation, but little is known about changes to the composition of PGs in response to lung infection or injury. The goals of this study were to identify changes to heparan sulfate PGs in a mouse model of gram-negative pneumonia, to identify the Toll-like receptor adaptor molecules responsible for these changes, and to determine the role of the heparan sulfate PG in the innate immune response in the lungs. We treated mice with intratracheal LPS, a component of the cell wall of gram-negative bacteria, to model gram-negative pneumonia. Mice treated with intratracheal LPS had a rapid and selective increase in syndecan-4 mRNA that was regulated through MyD88-dependent mechanisms, whereas expression of several other PGs was not affected. To determine the role of syndecan-4 in the inflammatory response, we exposed mice deficient in syndecan-4 to LPS and found a significant increase in neutrophil numbers and amounts of CXC-chemokines and total protein in bronchoalveolar lavage fluid. In studies performed *in vitro,* macrophages and epithelial cells treated with LPS had increased expression of syndecan-4. Studies performed using BEAS-2B cells showed that pretreatment with heparin and syndecan-4 decreased the expression of CXCL8 mRNA in response to LPS and TNF-α. These findings indicate that the early inflammatory response to LPS involves marked up-regulation of syndecan-4, which functions to limit the extent of pulmonary inflammation and lung injury.

Keywords: innate immunity; macrophages; neutrophils; proteoglycan; syndecan

Lung infections place a much higher burden on public health than better recognized diseases such as HIV/AIDS, cancer, heart attacks, and stroke (1). The innate immune system is the body's first line of defense against lung infection (2, 3). LPS, a component of the cell wall of gram-negative bacteria, is used to study lung infection and injury caused by gram-negative bacteria because it results in a potent and reproducible activation of the innate immune system (4, 5). Recognition of LPS by the innate immune system occurs via the Toll-like receptor (TLR)4/MD-2/CD14 receptor complex (2, 6). Upon binding LPS, TLR4 signals through two adaptor molecules, MyD88 and TRIF, which are required for the pulmonary inflammatory response and clearance of gram-negative bacteria from lungs (7, 8). An essential step in the clearance of most gram-negative bacteria from lungs is the pulmonary recruitment of neutrophils, whose influx is mediated by CXC chemokines, such as CXCL8 in humans and macrophage inflammatory peptide (MIP)-2 and KC in rodents (9–11). Although neutrophils are an important component of pulmonary host defenses, in excess numbers they contribute to tissue damage, leading to lung injury (12–14).

Once thought to be the molecular glue that provides structural support and imparts biomechanical properties to lung tissue, it is now recognized that proteoglycans (PGs) are important biological modifiers that regulate a number of processes such as tissue inflammation, lung development, homeostasis, and wound healing (15–17). Growing evidence supports important roles for PGs in the innate immune response to lung infection (17). Heparan sulfate (HS) is the most abundant glycosaminoglycan (GAG) in healthy lungs, and HS proteoglycans (HSPGs) play a key role in modulating tissue inflammation (16, 18). The binding of the CXC chemokines CXCL8, KC, and MIP-2 to GAGs such as HS in lungs provides fine-tune control of chemokine gradient formation and neutrophil migration (19, 20). The binding of chemokines and cytokines to GAGs protects them from proteolysis, thereby increasing their stability, which is thought to promote the inflammatory response in patients with cystic fibrosis (21–23). In addition, the shedding of syndecan-1, a cell-surface HSPG, plays an important role in regulating neutrophil influx into lungs in animal models (24, 25).

Changes in the composition of GAGs and PGs in lungs have been reported in animal models and human lung disease (26–30). After the treatment of lungs with LPS, the composition of GAGs changes from the healthy lung where HS predominates to inflamed lungs where chondroitin sulfate is the predominant GAG (26, 27). The changes that occur to HSPGs during the early stages of gram-negative pneumonia are not known. Therefore, identifying these changes and characterizing how they alter the inflammatory response could lead to a better understanding of the mechanisms controlling the innate immune response and the development of lung injury in patients with gram-negative pneumonia.

(*Received in original form August 24, 2011 and in final form March 8, 2012*)

This work was supported by National Institutes of Health grants HL098067, GM37696, RR030249, HL082658, HL081764, and DK089507 and by the Medical Research Service at the Department of Veterans Affairs and the Diffuse Lung Diseases Research Group from the Ministry of Health, Labor and Welfare, Japan.

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Am J Respir Cell Mol Biol Vol 47, Iss. 2, pp 196–202, Aug 2012

Originally Published in Press as DOI: 10.1165/rcmb.2011-0294OC on March 15, 2012
Internet address: www.atsjournals.org

The results provide evidence that expression of syndecan-4, a cell surface HSPG, was rapidly and selectively increased in response to LPS via MyD88-dependent signaling pathways. Experiments performed with syndecan-4–null (*Sdc4*$^{-/-}$) mice showed that the lack of this HSPG resulted in a significant increase in pulmonary inflammation and injury after treatment with LPS. Finally, studies performed *in vitro* showed that macrophages and epithelial cells increase the expression of syndecan-4 in response to treatment with LPS and that pretreatment with heparin and syndecan-4 decreased the expression of CXCL8 mRNA in a human epithelial cell line treated with LPS and TNF-α. These findings suggest that syndecan-4 has a critical role in the early inflammatory response in lungs by limiting tissue inflammation and injury.

MATERIALS AND METHODS

Reagents

The reagents used in this study were *Escherichia coli* serotype 0111:B4 LPS (List Biological Laboratories, Campell, CA or Sigma-Aldrich, St. Louis, MO), TaqMan primer-probes for quantitative PCR (Applied Biosystems, Foster City, CA), human TNF-α (Sigma-Aldrich), BEAS-2B cells (ATCC, Manassas, VA), human syndecan-4 (R&D, Minneapolis, MN), and low-molecular-weight heparin (Fragmin Pfizer, Tokyo, Japan).

Animal Protocols

The Animal Research Committees of the Veterans Affairs Puget Sound Health Care System and the Fukushima Medical University approved all animal experiments. C57BL/6 and C57BL/6J-*Ticam1Lps2*/J (TRIF mutant) mice (Jackson Laboratories, Bar Harbor, ME), MyD88 knockout (*MyD88*$^{-/-}$) (Dr. S. Akira at University of Osaka), and *Sdc4*$^{-/-}$ mice (Dr. T. Kojima) were housed in specific pathogen-free facilities. The intratracheal injection of LPS (1 μg/g), animal death at specified times, bronchoalveolar lavage (BAL), total and differential cell counts, ELISA for murine KC and MIP-2 (R&D) and measurement of total protein (Thermo Scientific, Rockford, IL) were performed as described (20, 31, 32).

Isolation of RNA

RNA was isolated with Absolute RNA Miniprep Kit (Stratagene, La Jolla, CA). Genomic DNA was digested with DNase I (Ambion, Austin, TX), and RNA was reverse transcribed with a High Capacity cDNA Archive Kit (Applied Biosystems).

Measurement of mRNA

Quantitative PCR was performed using TaqMan primer probes or Power SYBR Green PCR master mix and an ABI PRISM 7000 (Applied Biosystems) (32). The threshold cycle (Ct) was calculated using threshold cycles for the target genes and 18-S. Relative mRNA expression was expressed as fold increase over values obtained from RNA from normal lungs, untreated cells, or human reference total RNA (Stratagene).

Cell Culture

Bone marrow–derived macrophages (BMDMs) were cultured in macrophage medium (RPMI 1640, 10% FBS, 30% L929 cell supernatant, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/ml streptomycin) as described (20). After being cultured in macrophage medium for 6 days, BMDMs were isolated, counted, and cultured in macrophage media for 24 hours and then stimulated with LPS (10 or 100 ng/ml), IL-4/IL-13 (10 ng/ml), IL-10 (10 ng/ml), or RPMI 1640 for up to 48 hours. Alveolar macrophages isolated with repeated BAL using PBS were cultured for 24 hours in macrophage media and then stimulated with LPS for 4 hours. BEAS-2B cells were cultured in RPMI 1640 supplemented with 10% BSA, penicillin, and streptomycin for 5 to 6 days until they reached 90% confluence. The culture medium was then replaced, and low-molecular-weight heparin, syndecan-4, or RPMI 1640 was added for 1 hour. Cells were washed with PBS and incubated with LPS (1,000 ng/ml), TNF-α (1 ng/ml), or RPMI 1640 without heparin or syndecan-4 for 3 hours. Mouse tracheal air–liquid interface cultures were performed as described and stimulated with LPS or RPMI 1640 for 4 and 24 hours (33). Supernatants were removed and RNA was harvested.

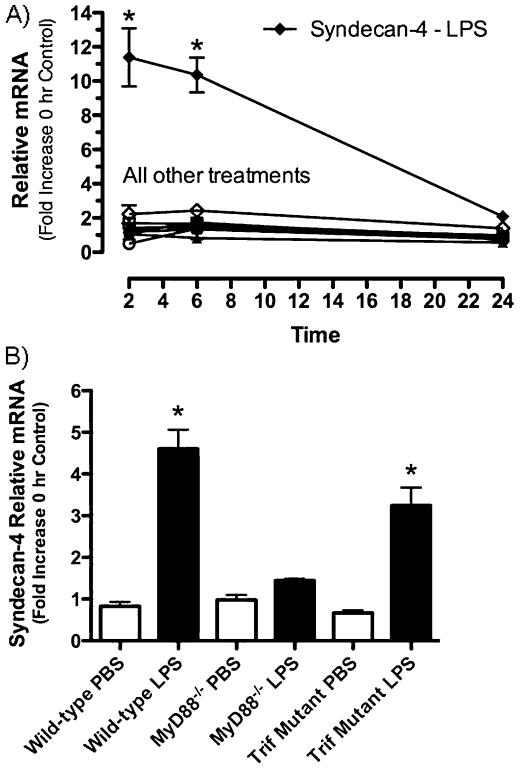

Figure 1. Changes in the relative amounts of mRNA for the heparan sulfate proteoglycans syndecan-1, -2, and -4 and perlecan, were determined using mRNA collected from whole lung homogenates and quantitative real-time PCR. Comparison of mRNA recovered from lungs of mice treated with PBS (*open symbols*) and LPS (*closed symbols*) were made at 2, 6, and 24 hours (*A*). Expression of syndecan-4 in whole lung homogenates collected from C57BL/6 (WT), MyD88 knockout (MyD88 KO), and Trif mutant mice (Trif mut) treated with PBS (*open bars*) or LPS (*closed bars*) for 2 hours (*B*). Values are the mean ± SEM with a minimum $n = 3$ for each group studied. The expression of mRNA for each proteoglycan studied is expressed as a relative fold increase in mRNA over the 0-hour control group. *Groups that are significantly different ($P \leq 0.05$) when mice treated with PBS and LPS were compared. The Mann-Whitney U-test was used for comparisons between the two treatment groups (*A*) and the Kruskal-Wallis test with Dunn's multiple comparison test was performed when multiple comparisons were made (*B*).

Flow Cytometric Analysis

BMDMs were detached, resuspended, incubated with Fc block (BD Pharmingen, San Diego, CA), and centrifuged at 1,500 rpm for 5 minutes at 4°C. The supernatant was removed, and BMDMs were resuspended in 100 μl binding buffer (PBS and 1% BSA) containing phycoerythrin-conjugated syndecan-4 antibody. After 1 hour, BMDMs were washed twice and analyzed using the Guava System (Millipore, Billerica, MA).

Statistical Analysis

Comparisons between multiple groups were performed using one-way ANOVA with Bonferroni's multiple comparison test or the Kruskal-Wallis test and Dunn's multiple comparison test. Student's *t* test and

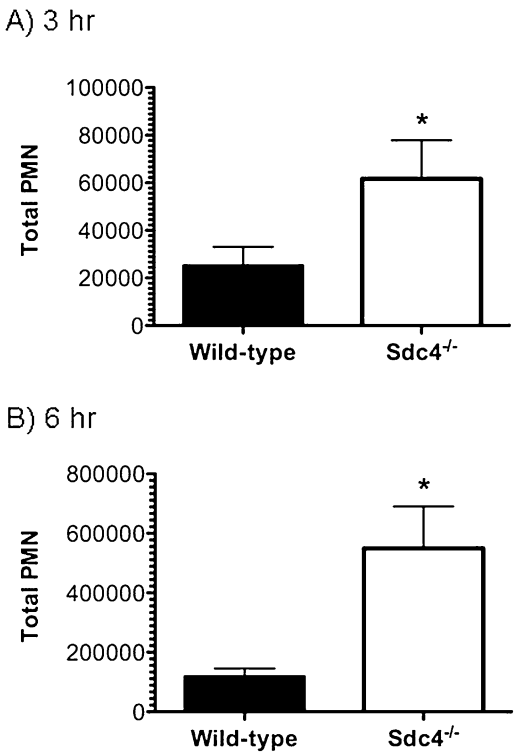

Figure 2. The total number of neutrophils recovered in bronchoalveolar lavage fluid collected from wild-type and syndecan-4 knockout ($Sdc4^{-/-}$) mice treated with LPS (1 μg/g) and followed for 3 hours (*A*) and 6 hours (*B*) was determined. Values are the mean ± SEM with $n = 8$ to 10 mice per group for the 3-hour study and $n = 11$ mice per group for the 6-hour study. The *y* axis differs on the graphs displaying the results for the 3- and 6-hour studies. *Significantly different using the Mann-Whitney U test and a $P \leq 0.05$.

the Mann-Whitney U test where used for comparisons between two groups. For all analyses, $P \leq 0.05$ was accepted as significant. The values shown are means ± SEM.

RESULTS

Measurement of mRNA for HSPGs in Whole Lung Homogenates

To characterize changes in the expression of mRNA for the HSPGs perlecan, syndecan-1, syndecan-2, and syndecan-4, mice were treated with LPS, and quantitative real-time PCR was performed using mRNA collected from whole lung homogenates. This work showed that, of the four HSPGs studied, only syndecan-4 mRNA was significantly increased after intratracheal LPS (11.4 ± 1.7-fold and 10.4 ± 1.7-fold at 2 and 6 h, respectively) when compared with control mice (Figure 1A). These findings show that among the HSPGs studied, syndecan-4 is rapidly and selectively up-regulated in response to LPS.

We next investigated the signaling pathways responsible for the increased expression of syndecan-4. The binding of LPS to the TLR4/MD2/CD14 complex results in signaling through two adaptor molecules, MyD88 and Trif (34). To evaluate the roles of these pathways in regulating the expression of syndecan-4 in lungs, mRNA was obtained from whole lung homogenates collected from C57BL/6 (i.e., wild-type controls), MyD88-null ($MyD88^{-/-}$) mice, and Trif mutant mice. Mice were killed 2 hours after treatment with LPS to minimize the chance that cytokines or growth factors produced in response to the LPS would indirectly increase the expression of syndecan-4 mRNA. The results showed that syndecan-4 mRNA was significantly increased in the wild-type and Trif mutant mice treated with LPS but not in the mice lacking MyD88 (Figure 1B). Thus, the increased expression of syndecan-4 in response to LPS was mediated via MyD88-dependent signaling pathways.

Measurement of the Innate Immune Response in Mice Deficient in Syndecan-4

To identify the role of syndecan-4 in modulating the pulmonary recruitment of neutrophils and the development of lung injury, wild-type mice and mice deficient in syndecan-4 ($Sdc4^{-/-}$ mice) were studied 3 and 6 hours after treatment with intratracheal LPS. We recovered significantly more neutrophils and greater amounts of the neutrophil chemotactic factors KC and MIP-2 from the airspaces of the $Sdc4^{-/-}$ lungs at 3 and 6 hours compared with wild-type control mice (Figures 2 and 3). Consistent with enhanced neutrophil influx leading to more injury, $Sdc4^{-/-}$ mice had a significant increase in total protein in the BAL fluid (Figure 4). These findings indicate that syndecan-4 plays an important role in the innate immune response to LPS by limiting the inflammatory response and lung injury in mice treated with LPS.

Measurement of Syndecan-4 mRNA in Macrophages and Epithelial Cells Treated with LPS

We assessed which cells in the lungs were responsible for the increased expression of syndecan-4. Immunohistochemistry of

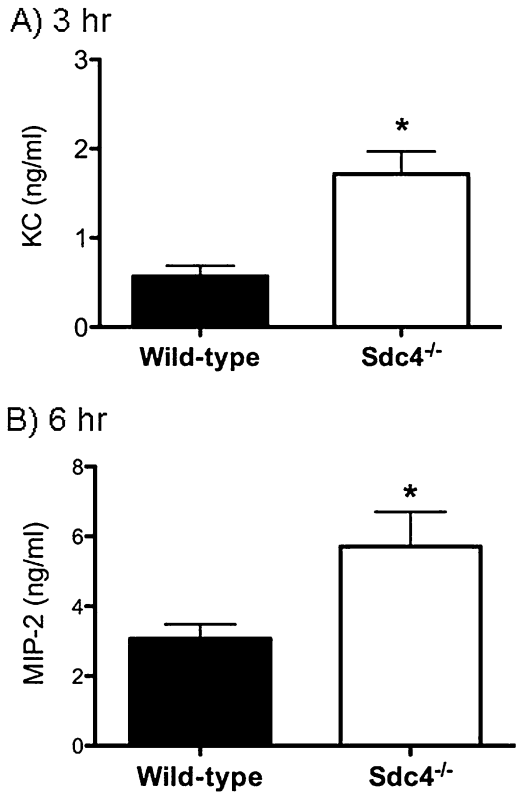

Figure 3. The amount of two neutrophil chemotactic factors, KC (*A*) and MIP-2 (*B*), was measured in the bronchoalveolar lavage fluid collected from wild-type and syndecan-4 knockout ($Sdc4^{-/-}$) mice treated with LPS (1 μg/g) and followed for 6 hours. Values are the mean ± SEM with $n = 8$ to 10 mice per group for the 3-hour study and $n = 11$ mice per group for the 6-hour study. *Significantly different using the Mann-Whitney U test and ($P \leq 0.05$).

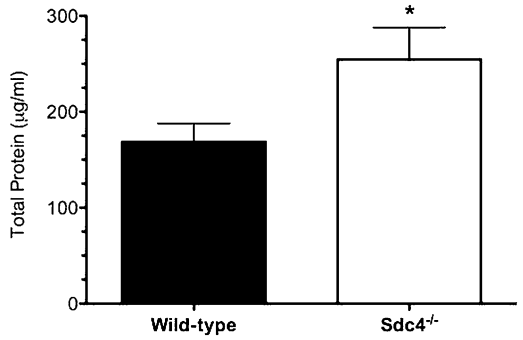

Figure 4. The amount of total protein in the bronchoalveolar lavage fluid collected from wild-type and syndecan-4 knockout (*Sdc4*$^{-/-}$) mice treated with LPS (1 μg/g) was measured at 6 hours. Values are the mean ± SEM with $n = 11$ mice per group. *Significantly different using the Mann-Whitney's U test and ($P \leq 0.05$).

tissues from *Sdc4*$^{-/-}$ mice with commercial antibodies demonstrated that none was specific for this antigen (data not shown). Thus, we used cell culture models to identify the cell types in lungs that might be responsible for the increased expression of syndecan-4. Macrophages and lung epithelial cells were chosen for these studies because they are the first cells exposed to an airway challenge of LPS and because both cell types express the TLR4/MD2/CD14 complex required to recognize and respond to LPS (35–38). For studies where mRNA was collected to measure alterations in syndecan-4 expression in macrophages, BMDMs were used for most experiments because of the numbers of cells required to make direct comparisons among the different treatments, which included two concentrations of LPS (10 and 100 ng/ml). The combination of IL-4/IL-13 or IL-10 alone was used for 4, 24, and 48 hours. LPS was used to differentiate cells into classically activated macrophages (M1), whereas IL-4/IL-13 in combination or IL-10 alone was used to differentiate these cells into alternatively activated macrophages (M2a and M2c, respectively) (39–41). The expression of syndecan-4 mRNA by macrophages was significantly increased after treatment with LPS but not with IL-4/IL-13 or IL-10 (Figure 5A), suggesting that this HSPG is a new marker of M1 macrophages. Compared with 0-hour control macrophages, syndecan-4 mRNA increased 41 ± 21-fold at 4 hours with 10 ng/ml LPS and increased 45 ± 28-fold with 100 ng/ml LPS. By 24 hours, the amount of syndecan-4 mRNA expression in macrophages treated with LPS had returned to control levels. In a limited number of experiments, murine alveolar macrophages were studied to confirm that these macrophages also increased the expression of syndecan-4 mRNA after treatment with LPS. These studies show that alveolar macrophages treated with LPS (100 ng/ml) for 4 hours had a 96.8 ± 6.8-fold increase in syndecan-4 mRNA (Figure 5B).

We next used flow cytometry to measure the amount of syndecan-4 present on the surface of BMDMs. Consistent with the increased expression of syndecan-4 mRNA (Figure 5A), macrophages treated with LPS had a significant increase in the amount of syndecan-4 measured on their cell surface (Figures 5C and 5D). Control macrophages had a mean fluorescence of 11.56 ± 0.19, whereas cells treated with LPS had a mean fluorescence of 129.2 ± 6.38. Confirmatory experiments using antibodies for syndecan-4 and the macrophage marker F4/80 showed that the syndecan-4–positive cells were macrophages (data not shown).

We also found that syndecan-4 expression by epithelial cells was increased by LPS (Figure 6) but not to the degree seen in macrophages. Murine tracheal epithelial cells grown at an air–liquid interface and treated with LPS at concentrations of 10 and 100 ng/ml for 4 hours had a 3.2 ± 0.74-fold and a 5.8 ± 0.85-fold increase in syndecan-4 mRNA, respectively (Figure 6A). In comparison when the human bronchial epithelial cell line BEAS-2B cells were treated with LPS (1,000 ng/ml), there was a 5.87 ± 1.53-fold and 3.94 ± 1.35-fold increase in

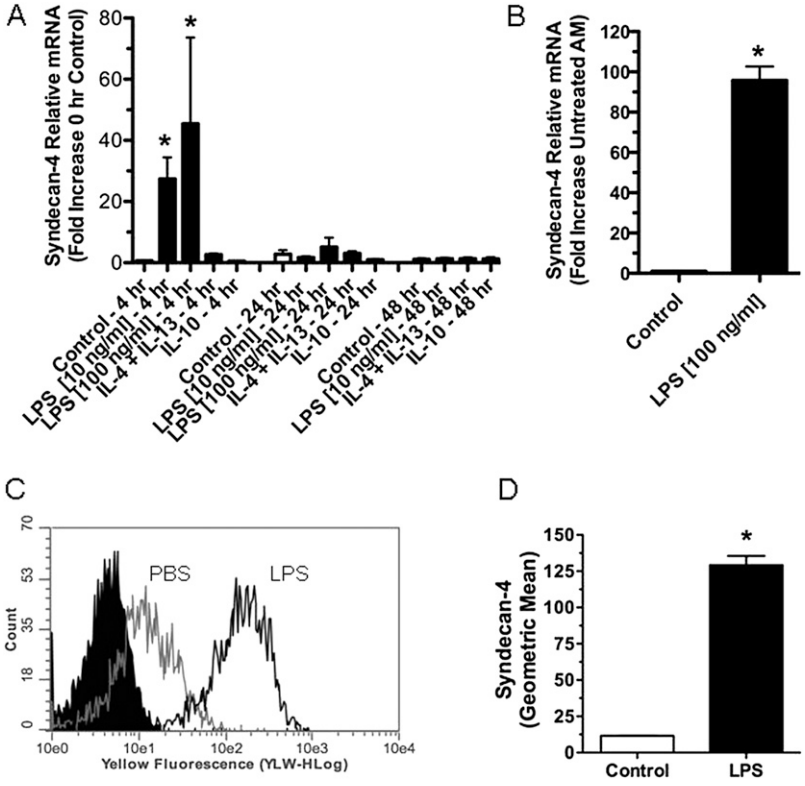

Figure 5. (*A*) Quantitative real-time PCR showing the relative expression for syndecan-4 mRNA collected from bone marrow–derived macrophages (BMDMs) treated *in vitro* with RPMI media (control), two concentrations of the M1 agonist LPS (10 and 100 ng/ml), the M2a agonists IL-4/IL-13 (10 ng/ml), or the M2c agonist IL-10 for up to 48 hours ($n = 4$–13). (*B*) Quantitative real-time PCR showing the relative expression for syndecan-4 mRNA collected from alveolar macrophages treated with RPMI media (control) or LPS (100 ng/ml) for 4 hours ($n = 4$). (*C* and *D*) A representative histogram (*C*) and the geometric mean of syndecan-4 expression (*D*) on BMDMs measured with flow cytometry. Flow cytometry was performed 3 hours after the treatment of mouse BMDMs with RPMI or LPS (10 ng/ml) ($n = 3$). Values are the mean ± SEM. *Groups different from the control mice ($P \leq 0.05$) using the Kruskal-Wallis test with Dunn's multiple comparison test (*A*) and the Mann-Whitney U test (*B* and *D*).

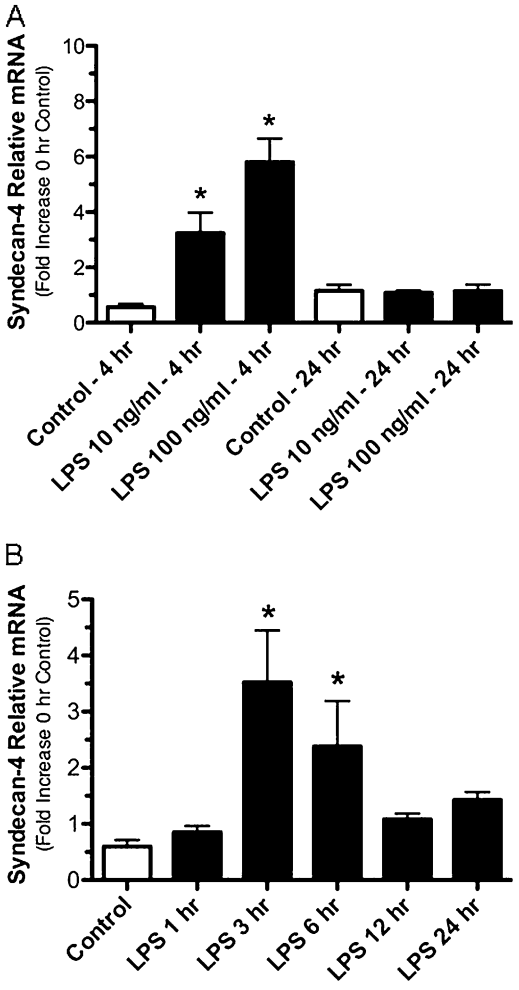

Figure 6. Quantitative real time PCR showing the relative expression for syndecan-4 mRNA collected from murine tracheal air–liquid interface cell cultures treated with LPS at 10 and 100 ng/ml (*A*) and BEAS-2B cells treated with LPS at 1,000 ng/ml (*B*) for the specified times. For all cultures, $n = 3$; for BEAS-2B cells, $n = 4$. Values are the mean ± SEM. *Groups different from the controls using the Kruskal-Wallis test with Dunn's multiple comparison test with $P \leq 0.05$.

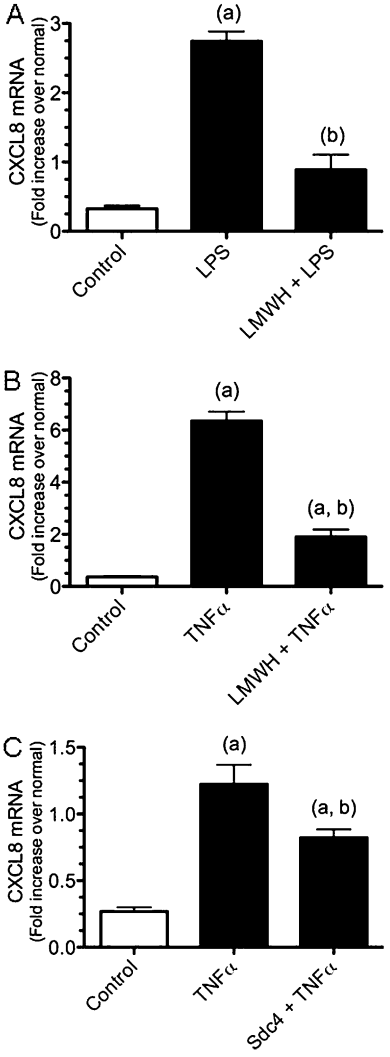

Figure 7. (*A* and *B*) Pretreatment of BEAS-2B cells with RPMI media or low-molecular-weight heparin (LMWH) (10 μg/ml) for 1 hour followed by treatment with media, LPS (*A*), or TNF-α (*B*) for 3 hours. (*C*) Pretreatment of BEAS-2B cells with RPMI media or syndecan-4 (2.5 μg/ml) for 1 hour followed by treatment with media or TNF-α for 3 hours. Values are the mean ± SEM with $n = 6$. An (a) shows groups that are different from the control group, and a (b) shows differences between LPS or TNF-α treatment groups using the Kruskal-Wallis test with Dunn's multiple comparison test with $P \leq 0.05$.

syndecan-4 mRNA after treatment for 3 and 6 hours, respectively. Taken together, these findings show that LPS causes a rapid up-regulation of syndecan-4 mRNA in macrophages and epithelial cells. In addition, we show that the rapid increase in the expression of syndecan-4 is a characteristic of classically activated macrophages.

Effect of Low-Molecular-Weight Heparin or Syndecan-4 on CXCL8 Expression in BEAS-2B Cells

We assessed if soluble forms of heparin or syndecan-4 alter the inflammatory response of BEAS-2B cells treated with LPS or TNF-α, a heparin-binding proinflammatory cytokine. Heparin was used for these studies because it is readily available and because it has a similar, though more sulfated, GAG backbone to heparan sulfate (18). BEAS-2B cells treated with LPS alone had significantly increased CXCL8 mRNA production compared with control cells (no LPS) (8.5 ± 0.45-fold increase versus control cells). However, when BEAS-2B cells were pretreated with low-molecular-weight heparin (LMWH) and then treated with LPS, these cells expressed significantly less CXCL8 mRNA (2.8 ± 0.67 for cells) as compared with cells treated with LPS alone (Figure 7A).

To determine if pretreatment with heparin would affect the activation of epithelial cells stimulated with the heparin-binding cytokine TNF-α (42, 43), BEAS-2B cells were pretreated with RPMI or LMWH and then treated with TNF-α. These studies showed that the preincubation of epithelial cells with LMWH significantly decreased the TNF-α induced up-regulation of CXCL8 mRNA expression (Figure 7B). Finally, to determine if syndecan-4 would affect the activation of epithelial cells stimulated with TNF-α, BEAS-2B cells were preincubated with human recombinant syndecan-4 (2.5 μg/ml) before treatment with TNF-α. The treatment of epithelial cells with recombinant syndecan-4 significantly inhibited the TNF-α induced up-regulation of CXCL8 mRNA in BEAS-2B cells (Figure 7C). As was observed with the LMWH, syndecan-4 did not completely abolish the TNF-α activation of BEAS-2B cells. These studies showed that pretreatment with heparin and HS are able to decrease the inflammatory response of epithelial cells subsequently treated with LPS and TNF-α *in vitro*.

DISCUSSION

The purpose of this work was to identify changes in the composition of HSPGs in mice exposed to gram-negative bacterial products and to determine how these changes alter pulmonary inflammation. To reduce animal-to-animal variability resulting

from live bacteria, mice were treated with intratracheal LPS, a component of the cell wall of gram-negative bacteria. The treatment of mice with intratracheal LPS resulted in a rapid increase in syndecan-4 mRNA in lungs that was regulated through MyD88-dependent signaling. Work performed using *Sdc4*$^{-/-}$ mice showed that the lack of this HSPG resulted in a significant increase in pulmonary inflammation and injury after treatment with LPS. Finally, experiments performed *in vitro* showed that macrophages and epithelial cells increase the expression of syndecan-4 in response to treatment with LPS and that pretreatment with heparin and syndecan-4 decreased the expression of CXCL8 mRNA in a human epithelial cell line treated with LPS and TNF-α. These studies show that syndecan-4 plays a key role in regulating the early innate immune response to lung infection.

Little is known about how the composition of HSPG changes during gram-negative pneumonia or about the signaling pathways responsible for these changes. Of the four HSPG examined, only the expression of syndecan-4 increased in mice treated with LPS. The rapid increase in syndecan-4 mRNA in lungs, which occurs in a similar time frame to the increased expression of proinflammatory cytokines and chemokines (44), suggests that the expression of this HSPG in mice exposed to intratracheal LPS is regulated directly through TLR4. To better characterize the signaling pathways responsible for the increased expression of syndecan-4, studies were performed in mice deficient in MyD88 or Triff. The inability of the *MyD88*$^{-/-}$ mice to increase syndecan-4 mRNA in response to LPS shows that the recruitment of MyD88 to TLR4 mediates signal transduction to downstream signaling components, such as NF-κB, which modulates syndecan-4 production (Figure 1B). This extends the previous work of Smith and colleagues who found that signaling through NF-κB was responsible for the increased expression of syndecan-4 in a gastric epithelial cell line treated with TLR2 and TLR4 agonists *in vitro* (45).

MyD88 is an adaptor molecular common to all TLRs (except for TLR3- and for IL-1R1) (3). Therefore, it is likely that syndecan-4 will be increased in a number of infectious and noninfectious lung diseases, with the common link being activation of MyD88 signaling pathways. For example, IL-1R1/MyD88 signaling and the inflammasome are essential in the development of pulmonary inflammation and fibrosis in bleomycin- induced lung injury (46), suggesting that IL-1R1/MyD88 signaling may be responsible for the increased expression of syndecan-4 reported in mice treated with bleomycin (47). These findings suggest an important role for syndecan-4 in regulating the inflammatory response in lungs after the activation of MyD88 signaling pathways.

To determine the role of syndecan-4 in the innate immune response to LPS, we studied mice lacking syndecan-4. This work showed that *Sdc4*$^{-/-}$ mice had increased pulmonary inflammation and lung injury after treatment with LPS. These results are consistent with previous reports, such as the work of Ishiguro and colleagues, who found that the injection of LPS into the peritoneum of *Sdc4*$^{-/-}$ mice resulted in increased mortality (48). In a more recent study, Jiang and colleagues showed that *Sdc4*$^{-/-}$ mice treated with bleomycin had increased pulmonary fibrosis (47). In addition, studies performed *in vitro* with BEAS-2B cells showed that pretreatment with heparin or syndecan-4 decreased the expression of CXCL8 mRNA when the BEAS-2B cells were subsequently treated with LPS or TNF-α (Figure 7). Taken together, this body of work shows that syndecan-4 regulates the inflammatory response and suggests that syndecan-4 may play a key role in preventing the adverse consequences of tissue inflammation, such as lung injury, pulmonary fibrosis, and the systemic inflammatory response.

To identify potential cellular sources for the increased expression of syndecan-4, murine macrophages and a human bronchial epithelial cell line (BEAS-2B cells) were treated with LPS for up to 24 hours. Macrophages and epithelial cells were evaluated because these cells are exposed to the highest concentrations of LPS after an airway challenge. In response to treatment with LPS *in vitro*, macrophages and epithelial cells showed a rapid and significant increase in the expression of syndecan-4, suggesting that they are in part responsible for the increased expression of syndecan-4 mRNA. Whereas the *in vitro* studies were performed using macrophages and epithelial cells, other cells, such as fibroblasts and endothelial cells, are potential sources of the increased recovery of syndecan-4 mRNA obtained from whole lungs of mice treated with LPS.

Macrophages are broadly classified into two groups: M1 or classically activated macrophages and M2 or alternatively activated macrophages. To characterize whether the early increase of syndecan-4 was specific for M1 macrophages, cells were treated with LPS to differentiate them to a M1 phenotype or IL-4/IL-13 and IL-10 to differentiate them to a M2 phenotype (39, 40). This work showed that the rapid increase in syndecan-4 expression in macrophages occurs only with LPS treatment, which suggests that syndecan-4 is an early marker of M1 macrophages.

In summary, the results of this study show that syndecan-4 is rapidly increased in response to the intratracheal instillation of LPS with expression kinetics similar to that observed for cytokines and chemokines. These studies also show an increased pulmonary inflammatory response and lung injury in syndecan-4–deficient mice treated with intratracheal LPS. Finally, pretreatment of lung epithelial cells with heparin or syndecan-4 inhibits the proinflammatory properties of LPS and TNF-α. We conclude that the increased expression of syndecan-4 in lungs of mice exposed to intratracheal LPS is an important mechanism that regulates neutrophil recruitment and limits the extent of pulmonary inflammation and lung injury. This suggests that the development of therapeutic strategies to capitalize on the protective effects of syndecan-4 may provide treatments to minimize the adverse effects of tissue inflammation.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Timothy Birkland, Ph.D., Kathleen R. Braun, Gina Kiske. and Vivian Lee for excellent technical expertise and assistance.

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