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A novel lung injury animal model using KL-6-measurable human MUC1-expressing mice

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ABSTRACT

KL-6, an epitope of MUC1 mucin expressed on type II pneumocytes and bronchiolar epithelia in humans, is a sensitive serum marker for interstitial pneumonia. However, an *in vivo* model for KL-6 has not been established because no KL-6 epitope is expressed in animals other than humans and apes. To investigate whether KL-6 is detectable in human MUC1-expressing (hMUC1-exp) mice and whether KL-6 level reflects the degree of lung injury, we examined serum and bronchoalveolar lavage fluid (BALF) levels of KL-6 and surfactant protein-D (SP-D) in either lipopolysaccharide (LPS)- or bleomycin (BLM)-induced lung injury models. KL-6 was expressed on type II pneumocytes and bronchiolar epithelial cells in naïve hMUC1-exp mice. Serum KL-6 levels in these mice were comparable to those in humans, and KL-6 levels in BALF were significantly higher than those in sera. In the LPS model, KL-6 levels in sera and BALF were slightly increased, although SP-D levels were markedly increased. During the inflammatory phase in the BLM model, KL-6 levels in sera were greatly increased, but those in BALF were decreased. Serum KL-6 levels were positively correlated with BALF albumin levels, a representative marker for increased the alveolar-capillary permeability. SP-D levels in sera and BALF were significantly increased compared to the corresponding levels in the LPS model. The increase in serum KL-6 levels appeared to be associated with the disruption of alveolar-capillary barrier after BLM-induced lung injury. This hMUC1-exp mouse can be used for assessment of KL-6 *in vivo* during lung injury.

1. Introduction

Krebs von den Lungen-6 (KL-6) is an epitope on MUC1 mucin, which is expressed on type II pneumocytes and bronchiolar epithelia in humans [1]. KL-6 is a sensitive serum biomarker for interstitial pneumonia and has both diagnostic and prognostic value [2,3]. High KL-6 levels suggest interstitial pneumonia rather than alveolar pneumonia [4]. Serum KL-6 levels are useful for determining the diagnosis of different types of interstitial pneumonia [5]. In addition, higher serum KL-6 levels predict a poorer prognosis in patients with idiopathic pulmonary fibrosis (IPF) [6]. Therefore, serum KL-6 levels of patients with pulmonary disease are frequently measured, approximately more than two million times per year, covered by insurance, in Japan [3,7].

Despite its clinical importance, the basic functions of KL-6 have not been fully determined. Compared to other serum markers such as surfactant protein-D (SP-D), the mechanisms for KL-6 changes in lung injuries remain unclear. Animal models have been developed to explore the basic mechanisms of lung injury and lipopolysaccharide (LPS)- or bleomycin (BLM)-induced lung injury models in rodents have been used to evaluate the efficacy of drugs [8,9]. However, KL-6 levels could not be measured in animals because KL-6 is found only in human and apes, but not in rodents, and a KL-6-measureable experimental animal model has not been established. Thus, we focused on human MUC1-expressing (hMUC1-exp) mice established by Dr. M.A. Hollingsworth [10], because KL-6 is an epitope of human MUC1 and might be expressed in these mice.

The hMUC1-exp mice express similar levels of human MUC1 with a distribution pattern consistent with humans [10]. This model has been used to study cancer immunity because MUC1 is expressed aberrantly in many human epithelial malignancies [11,12]. However, KL-6 expression has not been evaluated in hMUC1-exp mice. We postulated that KL-6 could be detected in the lungs, sera and bronchoalveolar lavage fluid (BALF) of hMUC1-exp mice by immunohistochemistry or enzyme-linked immunosorbent assays (ELISA). We also evaluated changes of KL-6 levels after LPS- or BLM-induced lung injury in hMUC1-exp mice.

2. Methods

2.1. Animals

hMUC1-exp mice (C57BL/6 background) were kindly provided by Dr. M.A. Hollingsworth (University of Nebraska Medical Center,

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Omaha, NE) [10], and age- and sex-matched wild-type (Wt) C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). The hMUC1-exp mice express both mouse MUC1 and human MUC1 and do not overexpress human MUC1. The MUC1 transgene was not expressed in tissues that did not normally express mouse MUC1 [10]. All animal procedures were approved by the ethics committee of our institution, and guidelines for animal welfare based on proper conduct of animal experiments (Science Council of Japan, 2006) were strictly observed. (See online supplements).

2.2. Measurement of KL-6, albumin, tumor necrosis factor-α (TNF-α), and SP-D levels in sera and BALF

Mice were euthanized by CO_2 inhalation, and immediately dissected. Blood samples were collected from the inferior vena cava. Lungs were lavaged by 3 instillations of 0.5 ml of phosphate-buffered saline (PBS) via the trachea, and BALF was collected. Serum and BALF KL-6 levels were measured by ELISA-based Lumipulse KL-6 kit (Eidia, Tokyo, Japan), according to the manufacturer's protocol. Albumin, TNF-α, and SP-D concentrations were measured by ELISA kits from Shibayagi (Gunma, Japan), Bay Bioscience (Kobe, Japan), and Yamasa (Chiba, Japan), respectively, according to the manufacturer's protocols.

2.3. LPS-induced lung injury model

Mice were divided into the following 7 groups: 0 h, 3 h, 6 h, 24 h, 48 h, 72 h, and 96 h. After mice were anesthetized with intraperitoneal injections of pentobarbital, LPS (*Escherichia coli* O111:B4, Sigma–Aldrich, St. Louis, MO; diluted with sterile saline, 5 g/50 l/mouse) or the same volume of PBS for the 0 h group was administered intratracheally with a MicroSprayer and a high-pressure syringe (Penn-Century, Philadelphia, PA), according to previously described methods [13]. Samples were collected at various time points after LPS challenge.

2.4. BLM-induced lung injury model

Mice were anesthetized with intraperitoneal injections of pentobarbital, and BLM (Sigma–Aldrich, Ontario, Canada; 2 mg/kg/mouse in 100 l of PBS) or the same volume of PBS was administered intratracheally with a MicroSprayer and a high-pressure syringe on day 1. Samples were collected on days 1, 8, and 22 and were subjected to ELISA, immunohistochemistry, collagen analyses, and/or reverse transcriptase-PCR (RT-PCR).

2.5. Histology and immunohistochemistry

After collecting sera and BALF, lungs were fixed with 10% formalin, embedded in paraffin and cut into 5-μm sections. The tissue sections were stained with hematoxylin and eosin (H.E.) and/or Masson-trichrome staining. Lung fibrosis was quantified by using the Ashcroft scoring system [14]. KL-6 expression was evaluated by immunohistochemistry with a mouse anti-KL-6 IgG1 monoclonal antibody (mAb) (1 g/ml; kindly provided by Eidia), as previously reported [15]. A mouse IgG1 antibody (1 g/ml; Dako Denmark A/S, Glostrup, Denmark) was used as an isotype control.

2.6. Collagen assay

Collagen contents in the lungs were measured by a collagen assay kit (Sircol Soluble Collagen Assay, Biocolor, Carrickfergus, UK), according to the manufacturer's protocol. (See online supplements).

2.7. Semi-quantitative RT-PCR analysis for hMUC1 mRNA

Lung tissues were soaked in RNeasy solution (Qiagen, Venlo, Netherlands) at −80 °C until use, and total RNA from whole lung was extracted by a standard extraction kit. Two g of total RNA was reverse transcribed into cDNA by using a two-step Prime Script RT-PCR kit (Takara Bio, Shiga, Japan), according to the manufacturer's procedures. The cDNA was amplified by PCR and the products were electrophoresed on 2% agarose gels (See online supplements).

2.8. Western blot analysis of KL-6 and MUC1

Whole lung cell lysates were extracted from freshly frozen lungs by using standard methods. A total of 25 g of protein per lane was fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted on to PVDF membrane, and then reacted with anti-KL-6 mAb (kindly provided by Eidia), anti-hMUC1 mAb (Cell Signaling Technology, Boston, MA) or anti- actin mAb (AbCam, Cambridge, UK). Specific proteins were detected by a chemiluminescence method performed with horseradish peroxidase-conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch, West Grove, PA) and the ECL system (GE healthcare, Little Chalfont, UK) (See online supplements).

2.9. Statistical analysis

All data are expressed as mean ± SEM. The Mann–Whitney *U* test was used to determine differences between the controls and lung injury groups. The correlations between markers were calculated by using Spearman's rank correlation coefficient. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. KL-6 expression in naïve hMUC1-exp mice

In naïve hMUC1-exp mice, the KL-6 levels were 221 ± 18 U/ml in sera and 2121 ± 129 U/ml in BALF ($p < 0.001$). The KL-6 levels in Wt mice were under the detection limit. Immunohistochemistry with anti-KL-6 mAb revealed that Wt mice did not express KL-6. In naïve hMUC1-exp mice, KL-6 was mainly detected in type II pneumocytes and bronchiolar epithelial cells and partly detected in epithelial lining fluid (ELF) (Fig. 1); this distribution is similar to the distribution of hMUC1 expression in the respiratory system of hMUC1-exp mice [10] and that of KL-6 expression in human lungs [2].

3.2. LPS-induced lung injury model in hMUC1-exp mice

Morphologically, the inflammatory responses in LPS-induced lung injury were similar between hMUC1-exp mice and Wt mice and peaked at 6 h after LPS administration (Supplementary Fig. S1A). Total cells in BALF, mostly neutrophils, were significantly increased 6 h to 72 h after LPS administration (Supplementary Fig. S1B). However, no significant differences were seen in BALF cell counts or cell composition between hMUC1-exp mice and Wt mice. KL-6 expression levels on alveolar epithelial cells remained stable for up to 24 h after LPS challenge, as determined by immunohistochemistry (Supplementary Fig. S1C). Semi-quantitative RT-PCR revealed no significant changes in hMUC1 mRNA expression between 0 h, 24 h, and 96 h after LPS challenge (data not shown).

3.3. KL-6 levels in LPS-induced lung injury model

Serum KL-6 levels were gradually increased up to 1.8-fold of those at 0 h with a peak at 72 h after LPS administration (0 h versus

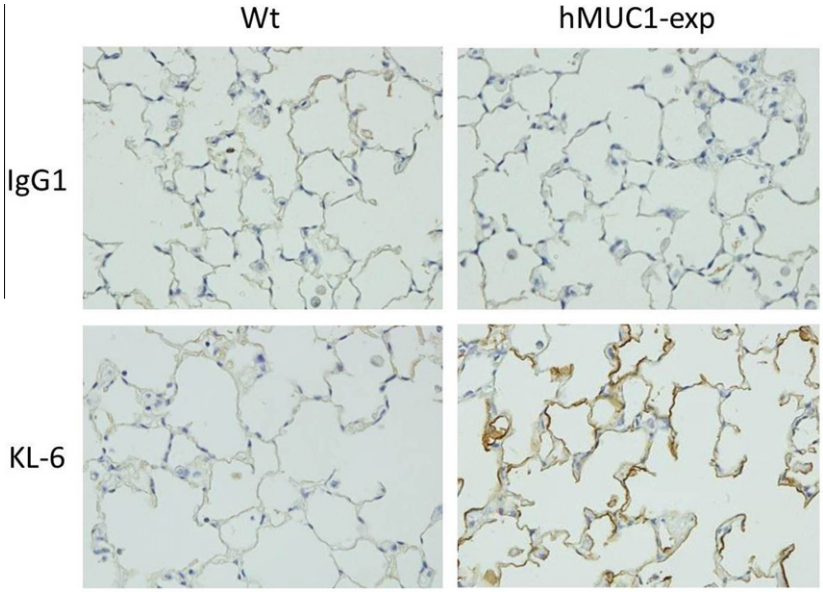

Fig. 1. KL-6 expression in naïve hMUC1-exp mice. Immunohistochemistry shows KL-6 expression on alveolar epithelial cells of hMUC1-exp mice, but not of wild type (Wt) mice. Upper and lower panels show IgG1 isotype control mAb staining and anti-KL-6 mAb staining, respectively (Original magnification, ×400).

72 h, $p = 0.008$) (Supplementary Fig. S2A). BALF KL-6 levels were also gradually increased with a peak at 72 h after LPS challenge (0 h versus 24 h, 48 h, 72 h, and 96 h, $p < 0.001$, = 0.002, < 0.001, = 0.002, respectively) (Supplementary Fig. S2B). The maximum levels of BALF KL-6 were approximately 1.6-fold higher than those at 0 h. There were no statistically significant changes in BALF albumin levels. Western blot analysis revealed stable expression of KL-6 and hMUC1. TNF- levels were dramatically increased in BALF, but not in sera, with a peak at 6 h after the administration of LPS. There were no significant differences in TNF- levels between hMUC1-exp mice and Wt mice (Supplementary Fig. S2C).

3.4. BLM-induced lung injury model in hMUC1-exp mice

In the BLM model, hMUC1-exp mice showed similar morphological inflammatory responses on day 8 and fibrotic responses on day 22 as compared with Wt mice, as determined by H.E. staining (Supplementary Fig. S3A). Total cell counts, especially lymphocyte counts, in BALF were increased on day 8 and 22 as compared to day 1 in both groups. There were no significant differences in BALF cell counts and cell composition between hMUC1-exp mice and Wt mice. Masson-trichrome staining of the lungs on day 22 showed similar fibrotic changes in hMUC1-exp mice and Wt mice (Supplementary Fig. S3A). Collagen assays showed no significant differences in increased soluble collagen contents on day 22 in the lungs of hMUC1-exp mice and Wt mice ($p = 0.65$) (Supplementary Fig. S3B). Likewise, there were no significant differences in lung fibrosis, as assessed by Ashcroft scores, in hMUC1-exp mice and Wt mice ($p = 0.38$) (Supplementary Fig. S3C). These results suggest that phenotypes of lung injury models were not different between hMUC1-exp mice and Wt mice and that hMUC1-exp mice can be used to monitor KL-6 in conventional lung injury models.

3.5. KL-6 and albumin levels in the BLM-induced lung injury model

As compared to the LPS model, KL-6 showed dramatic changes in both the sera and BALF of BLM-treated mice (Fig. 2A). On day 8, serum KL-6 levels were dramatically increased to approximately 10-fold higher than the day 1 levels in hMUC1-exp mice ($p < 0.001$); however, BALF KL-6 levels in BLM-treated mice were decreased to 0.77-fold of baseline levels ($p = 0.009$). On day 22, serum KL-6 levels were decreased compared with the levels on day 8, but were still higher than the levels on day 1 ($p = 0.006$). BALF KL-6 levels on day 22 were still 1.8-fold higher than those on day 1 ($p = 0.006$). These results suggest the possible leakage of KL-6 from alveolar spaces to the bloodstream by increased alveolar-capillary permeability during the inflammatory phase. To investigate the possible association of serum KL-6 levels and increased alveolar-capillary permeability, we measured albumin levels in BALF. Contrary to the changes of KL-6 levels in BALF, BALF albumin levels were significantly increased on day 8 (Fig. 2B). As shown in Fig. 2C, the time course of BALF KL-6 levels adjusted to albumin levels was similar to the pattern of that before adjustment, but showed a more drastic decrease in KL-6 levels on day 8. In addition, serum KL-6 levels were positively correlated with BALF albumin concentrations (Fig. 2D). These results clearly demonstrate that increased serum KL-6 levels on day 8 after BLM-induced lung injury are associated with increased leakage of albumin from capillaries to alveolar spaces, suggesting increased alveolar-capillary permeability.

3.6. Expression of KL-6 and hMUC1 in the BLM-induced lung injury model

Immunohistochemical studies of BLM-induced lung injury in hMUC1-exp mice revealed a partial lack of KL-6 expression on day 8 and much stronger KL-6 expression on day 22 as compared to the expression on day 1 (Fig. 3A), which is parallel to the changes of KL-6 levels in BALF. Wt mice and isotype negative control IgG1 staining showed no KL-6 expression. To determine whether the decrease in BALF KL-6 is due to decreased hMUC1 mRNA expression, semi-quantitative RT-PCR of hMUC1 was performed. Levels of hMUC1 mRNA were not significantly changed between day 1, day 8, and day 22 after standardization to *G3PDH* levels (Fig. 3B). Next, protein levels of KL-6 and human MUC1 were evaluated by Western blotting. KL-6 protein levels tended to increase on day 22, and human MUC1 showed significant increases on day 22 ($p < 0.01$) after standardization to β-actin levels (Fig. 3C).

3.7. SP-D levels in the LPS and the BLM models

In the LPS model, serum SP-D levels were significantly increased with a peak at 72 h after LPS administration (Fig. 4A). BALF SP-D

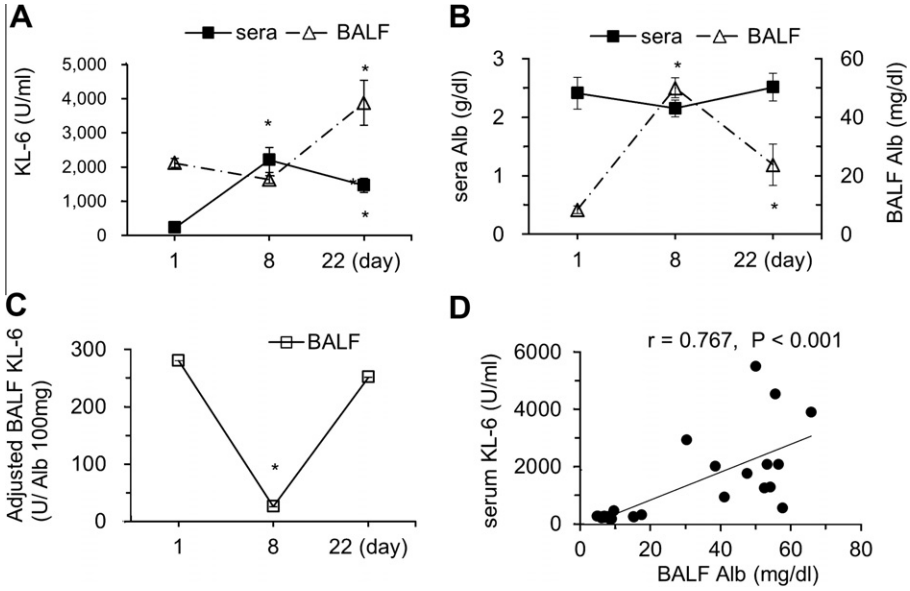

Fig. 2. Changes of biomarkers in BLM-induced lung injury model. KL-6 (A) and albumin (B) levels in sera and BALF in hMUC1-exp mice (n = 9–13). On day 8, serum KL-6 levels are dramatically increased to 10-fold higher than those observed at day 1 in hMUC1-exp mice. However, BALF KL-6 levels are decreased to 0.77-fold of baseline levels. KL-6 and albumin levels exhibited opposite changes in BALF. *p < 0.05, compared with day 1. (C) Albumin-adjusted BALF KL-6 levels show a similar pattern to that before adjustment. (D) Serum KL-6 levels are positively correlated with albumin level in BALF (n = 27).

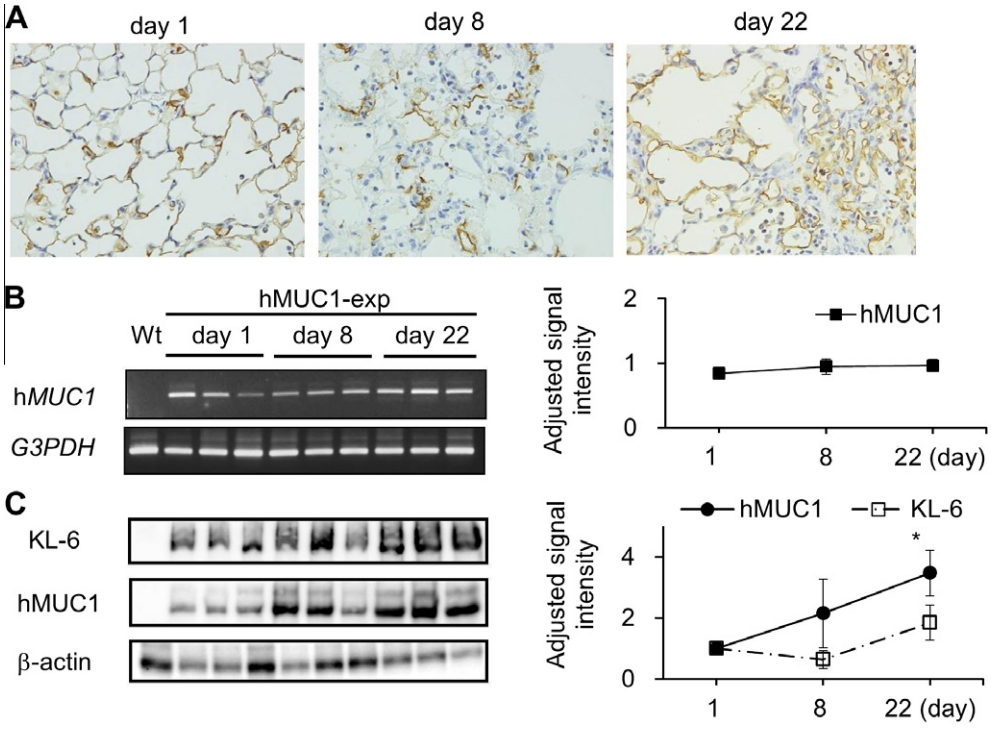

Fig. 3. Changes of KL-6 expression in BLM-induced lung injury model. (A) Immunohistochemistry showing a partial decrease of KL-6 expression in hMUC1-exp mice in the inflammatory phase (on day 8) and increased expression in the fibrotic phase (on day 22) as compared with the expression on day 1. (B) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of hMUC1 in hMUC1-exp mice shows no significant change on day 22 after standardization to *G3PDH* levels. In western blotting, KL-6 and MUC1 protein expression are increased gradually on day 8 and day 22, and the MUC1 expression increased more than the KL-6 expression.

levels were also significantly increased with a peak at 24–72 h after LPS administration (Fig. 4B). In the BLM model, SP-D levels were significantly increased in both the sera and BALF of Wt mice and hMUC1-exp mice (Fig. 4C and D). SP-D levels in sera and BALF were markedly elevated on day 8 and then sharply decreased on day 22. BALF SP-D levels were modestly decreased on day 22. There were no significant differences in SP-D levels between hMUC1-exp mice and Wt mice.

4. Discussion

The present study has demonstrated that KL-6 was detected in naïve hMUC1-exp mice by ELISA and immunohistochemistry. Surprisingly, their serum KL-6 levels were similar to the levels in healthy humans, which were generally less than 500 U/ml [2]. Immunohistochemistry revealed that KL-6 was mainly expressed in type II pneumocytes and partly expressed in ELF, which is

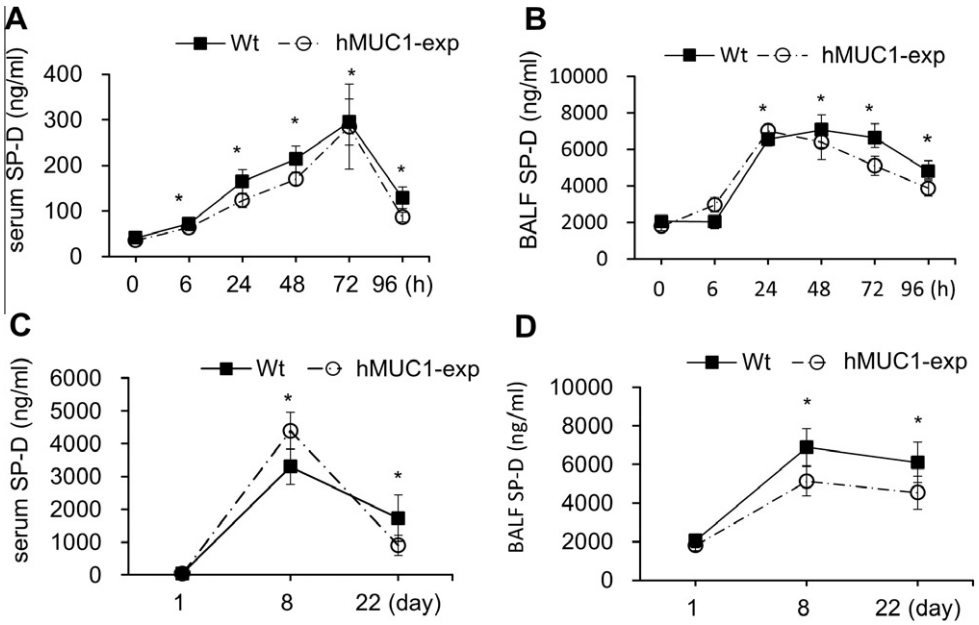

Fig. 4. Sequential changes in SP-D in the BLM model and the LPS model. In the LPS model, SP-D levels in sera (A) and BALF (B) are also significantly increased with a peak at 72 h after LPS challenge. There are no significant differences in SP-D levels between hMUC1-exp mice and Wt mice. $^{*}p < 0.05$, compared with 0 h. In the BLM model, SP-D levels in sera (C) and BALF (D) are significantly more increased than the corresponding levels in the LPS model. There are no significant differences in SP-D levels between hMUC1-exp mice and Wt mice.

similar to its expression in humans. These results suggest that KL-6 was produced mainly by type II pneumocytes in the hMUC1-exp mice, and that KL-6 would be a useful biomarker of lung injury in the animal model as well.

As expected, hMUC1-exp mice showed the same responses as conventional lung injury mouse models, different types of lung injury caused different changes to biomarkers in sera and BALF, and serum KL-6 levels reflected the degree of lung injury. In the LPS-induced acute lung injury model, hMUC1-exp mice and Wt mice developed similar rapid neutrophilic inflammation accompanied by rapid increases in the levels of TNF-α and SP-D, as previously reported [8]. KL-6 levels in sera and BALF showed modest but significant increases after LPS treatment. In the BLM-induced lung injury model, both hMUC1-exp mice and Wt mice showed similar inflammatory and fibrotic changes, including increased levels of SP-D and collagen, as previously reported [9]. KL-6 levels in sera were dramatically increased in the inflammatory phase (day 8) and then decreased in the fibrotic phase (day 22) but were still higher than the control levels. In contrast, BALF KL-6 levels were decreased on day 8 and then dramatically increased on day 22. Similarly, immunohistochemical studies demonstrated that KL-6 expression was partially decreased on day 8 and increased on day 22. The partial lack of KL-6 expression on day 8 might reflect the decreased KL-6 levels in ELF. These results demonstrated that hMUC1-exp mice could be used for the monitoring of KL-6 in conventional lung injury models.

Although the mechanisms for the transfer of KL-6 from alveolar epithelia to the bloodstream are unclear, our data suggest that increased alveolar-capillary permeability is one of the most logical explanations. KL-6 is thought to move from ELF to the bloodstream through capillaries after cleavage from MUC1 [2]. The disruption of the normal alveolar-capillary barrier is associated with acute lung injuries, and BALF albumin is a representative marker for such conditions [16,17]. Serum KL-6 levels were positively correlated with BALF albumin levels in the BLM model, suggesting that the leakage of KL-6 from ELF to the bloodstream and the leakage of albumin from the bloodstream to alveolar spaces are promoted by increased alveolar-capillary permeability. Another possible explanation for KL-6 changes could be the enhanced production of KL-6 from regenerated type II pneumocytes after alveolar wall injuries, as previously reported [18]. In fact, immunohistochemistry on day 22 after BLM treatment showed stronger KL-6 expression than on day 1 in hMUC1-exp mice. However, semi-quantitative RT-PCR resulted in no significant changes in hMUC1 mRNA expression in the BLM model. In addition, Western blot analysis showed a slight increase in KL-6 levels and a significant increase in MUC1 levels on day 22. In these experiments, we used total RNA or protein isolated from whole lung tissues, which contained a mixture of inflammatory cells, fibroblasts, and other cell types. We assumed that KL-6 and MUC1 signals in type II pneumocytes were diluted by signals from other types of cells after adjusting for housekeeping gene expression, despite the increased production of KL-6 and MUC1. Another explanation is that epigenetic mechanisms may play a crucial role in the production of KL-6 on MUC1 mucin. Although the specific enzyme involved in the glycosylation of the KL-6 epitope is unclear, a glycosyltransferase may be involved [19]. The other possibility is that the clearance of KL-6 through lymphatic channels may greatly influence BALF KL-6 levels [7,15,20]. Further studies are needed to examine these possible mechanisms for KL-6 transfer.

Levels of SP-D changed differently from those of KL-6 during lung injuries. SP-D levels in both sera and BALF were increased in the LPS or BLM models, as previously reported [21–23]. Similarly to KL-6 levels, serum SP-D levels were much higher in the BLM model than in the LPS model. According to previous reports [22,23], the primary mechanisms for increased levels of SP-D are epithelial barrier disruption and epithelial hyperplasia. Unlike the BLM model, no statistically significant differences in BALF albumin levels were noted in the LPS model, suggesting that the disruption of the alveolar-capillary barrier in the BLM model is much more severe than in the LPS model. Regenerated epithelia play an important role in producing these markers. Transient type II cell hyperplasia resulted in increased serum SP-D levels in a rat BLM-induced lung injury model [23]. Compared with SP-D, KL-6 showed a modest increase in the LPS model. This difference could be dependent on the differences in molecular sizes of these

biomarkers [7,24,25]. Large molecules (e.g. KL-6 >1000 kDa) require more severe disruption of permeability to leak out. In addition, cleavage from the cell membrane is necessary for KL-6 prior to its transfer. We speculate that once KL-6 is lost from ELF and alveolar cells, it might take more time to be recovered as compared to SP-D. Further studies are needed to understand the mechanisms for changes in KL-6 and SP-D levels.

In summary, we found that KL-6 can be measured in a hMUC1-exp mice model and that KL-6 levels were closely correlated with lung injuries in these mice. Therefore, this model is a promising tool for studying lung injuries and exploring the mechanisms of KL-6.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.123.

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