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GM-CSF mediates alveolar epithelial type II cell changes, but not emphysema-like pathology, in SP-D-deficient mice.

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Ochs, Matthias, Lars Knudsen, Lennell Allen, Amber Stumbaugh, Stacey Levitt, Jens R. Nyengaard, and Samuel Hawgood. GM-CSF mediates alveolar epithelial type II cell changes, but not emphysema-like pathology, in SP-D-deficient mice. Am J Physiol Lung Cell Mol Physiol 287: L1333–L1341, 2004. First published August 13, 2004; doi:10.1152/ajplung.00137.2004.—Surfactant protein D (SP-D) is a member of the collectin subfamily of C-type lectins, pattern recognition proteins participating in the innate immune response. Gene-targeted mice deficient in SP-D develop abnormalities in surfactant homeostasis, hyperplasia of alveolar epithelial type II cells, and emphysema-like pathology. Granulocyte/macrophage colony-stimulating factor (GM-CSF) is required for terminal differentiation and subsequent activation of alveolar macrophages, including the expression of matrix metalloproteinases and reactive oxygen species, factors thought to contribute to lung remodeling. Type II cells also express the GM-CSF receptor. Thus we hypothesized GM-CSF might mediate some or all of the cellular and structural abnormalities in the lungs of SP-D-deficient mice. To test this, SP-D (D/H11001) and GM-CSF (D/H11002) single knockout mice as well as double knockout mice were analyzed by design-based stereology. Compared with wild type, D/G+ as well as D/G− mice showed decreased alveolar numbers, increased alveolar sizes, and decreased alveolar epithelial surface areas. These emphysema-like changes were present to a greater extent in D/G− mice. D/G+ mice developed type II cell hyperplasia and hypertrophy with increased intracellular surfactant pools, whereas D/G− mice had smaller type II cells with decreased intracellular surfactant pools. In contrast to the emphysemaous changes, the type II cell alterations were mostly corrected in D/G− mice. These results indicate that GM-CSF-dependent macrophage activity is not necessary for emphysema development in SP-D-deficient mice, but that type II cell metabolism and proliferation are, either directly or indirectly, regulated by GM-CSF in this model.

The surfactant system of the lung prevents alveolar collapse by reducing alveolar surface tension. Growing evidence suggests that certain surfactant components also have immunomodulatory functions. Surfactant is a complex mixture of lipids and proteins, including the surfactant apoproteins SP-A, SP-B, SP-C, and SP-D (30). SP-D, the focus of this study, binds phosphatidylinositol, a minor surfactant phospholipid, but to date, a clear function for SP-D in surfactant homeostasis or function has not been established (reviewed in Ref. 17). SP-D, like SP-A, is a member of the collectin protein family. Collectins, proteins with extended collagen-like domains linked to C-type lectin domains, participate in the innate immune response by both binding a wide variety of glycoconjugates and modulating immune cell responses (reviewed in Refs. 7, 8, 18, 28, and 42). SP-D gene-targeted mice develop complex postnatal abnormalities in surfactant homeostasis leading to alveolar lipoproteinosis associated with alterations of alveolar type II cells and alveolar macrophages (6, 23, 25). Although qualitative findings suggest hypertrophy of type II cells with occasional formation of giant lamellar bodies in SP-D-deficient mice (6), an appropriate stereological analysis quantifying alterations in the number and size of type II cells and lamellar bodies has not been performed. SP-D-deficient mice also develop emphysema-like lesions with increased expression of matrix metalloproteinases (MMPs) (41) and increased production of reactive oxygen species (ROS) (45) and reactive nitrogen species (RNS) (2), suggesting an ongoing inflammatory process probably mediated by alveolar macrophages leading to destructive lung remodeling.

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a cytokine released by various cell types in response to inflammatory stimuli. Alveolar type II cells and alveolar macrophages express GM-CSF and both subunits of the GM-CSF receptor, and genetic experiments suggest a role for GM-CSF in the function of both these cell types (see Refs. 12, 33, 38, and 39 for review). Overexpression of GM-CSF in mice causes type II cell hyperplasia and an increase in the number of alveolar macrophages (20). Mice deficient in GM-CSF develop lung alterations reminiscent of pulmonary alveolar proteinosis with decreased surfactant clearance due to a block in surfactant degradation by alveolar macrophages (10, 22, 36, 44), but specific changes in lung architecture have not been described. GM-CSF-deficient mice show an increased susceptibility to pulmonary infections (27, 36). Together, there is growing evidence suggesting that GM-CSF is an important regulator of the alveolar epithelium, surfactant homeostasis, and lung host defense (reviewed in Refs. 38 and 39).

Studies in GM-CSF-deficient mice (3, 4, 35) and humans (5) demonstrated that GM-CSF is required for the expression of the transcription factor PU.1, which in turn is required for most of the differentiated functions of alveolar macrophages, includ-

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ing the expression of specific cell surface receptors involved in phagocytosis, MMP expression, ROS production, surfactant catabolism, and secretion of proinflammatory cytokines. GM-CSF is modestly increased in the lungs of SP-D-deficient mice (15), and there is indirect evidence for GM-CSF-dependent activation of alveolar macrophages, as shown by increased levels of MMPs (41) and ROS and RNS (2, 45). In other models of lung inflammation, macrophage activation, specifically MMP-12 expression, is associated with emphysema-like pathology (14, 29) similar to that seen in SP-D-deficient mice. Thus we hypothesized that suppression of GM-CSF-dependent alveolar macrophage functions would attenuate the lung structural abnormalities present in SP-D-deficient mice. To test this hypothesis, a quantitative phenotype analysis was performed by means of design-based stereology with emphasis on emphysema-like alterations of parenchymal architecture and alterations in the number and size of alveolar type II cells and their lamellar bodies and on the number and size of alveolar macrophages in mice deficient for SP-D, GM-CSF, and both SP-D and GM-CSF.

**MATERIALS AND METHODS**

**Transgenic mice.** SP-D-deficient mice were developed in an outbred C57Bl6/CD-1 background as previously described (6). GM-CSF-deficient mice fully backcrossed into a C57Bl6 background were obtained from Dr. Glenn Dranoff, Harvard University (10). Homozygous SP-D-deficient and GM-CSF-deficient mice were first bred to obtain mice heterozygous for both the SP-D and GM-CSF alleles. Double heterozygous mice were then intercrossed to produce study mice that were wild type (WT) for both genes, null for SP-D alone (D–G+) or GM-CSF alone (D–G–), or null for both genes (D–G–) (15). Littermates were used for this study to control for possible confounding effects of different genetic background. The SP-D-deficient line used was not fully backcrossed, so some genetic variability was still possible. Littermates were screened by sequential PCR reactions using primers specific for the SP-D- and GM-CSF-targeted alleles, respectively. Mice were kept in isolator cages in a barrier facility for the duration of this study. The Committee on Animal Research at the University of California San Francisco approved all experimental protocols.

**Fixation, sampling, and processing.** From each genotype (WT, D–G+, D–G–, D–G–), five animals aged 12 wk were analyzed. Lung fixation was performed by intratracheal instillation of 2% glutaraldehyde/1% freshly prepared paraformaldehyde in 0.1 M phosphate buffer at a pressure of 20 cmH2O. After storage of the lungs in fixative for at least 2 h, the total volume of the lungs was determined by fluid displacement (34). Systematic, uniformly random samples that, by definition, represent all parts of the whole organ equally well, were then taken and processed for analysis by light and electron microscopy as described previously (11, 16). For light microscopy, the sections were osmicated, bloc stained in aqueous uranyl acetate, dehydrated in ethanol, and embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer). For transmission electron microscopy, the sections were osmicated, bloc stained in aqueous uranyl acetate, dehydrated in acetone, and embedded in LX 112 (Ladd Research Industries, Burlington, VT).

**Stereological analysis.** At the level of light microscopy, stereological analysis was performed with an Axioskop light microscope (Zeiss, Oberkochen, Germany) equipped with a computer-assisted stereology system (CAST 2.0; Olympus, Ballerup, Denmark). Four glycol methacrylate and LX 112 blocks from each lung were analyzed. From each glycol methacrylate block, two sections of 1-μm thickness (the 1st and 4th of a consecutive row) were aligned in parallel on one glass slide. The sections were stained with methylene blue or orcein. By means of point and intersection counting on the first of the two adjacent sections, the volume fractions of parenchymal tissue, of air spaces within parenchymal tissue, and of septal tissue within parenchymal tissue as well as the surface area of the alveolar epithelium and the volume-to-surface area of alveolar septae were determined according to established methods (9, 19). The number of alveoli was determined by the estimation of the Euler number of the network of alveolar openings using the two adjacent sections as physical disectors (21, 31). The mean size of an individual alveolus was estimated by dividing total air space volume by alveolar number. From each LX 112 block, two (the 1st and 4th of a consecutive row) semithin sections of 1-μm thickness were mounted in parallel on one glass slide and stained with methylene blue. The number of type II cells per lung was estimated with the physical disector principle (37). The mean size of a single type II cell was estimated using the planar rotator (40). The number of alveolar macrophages per lung and the mean size of a single alveolar macrophage were estimated accordingly. For the size estimation of both cell types, global isotropy was assumed.

At the level of electron microscopy, the number of lamellar bodies per type II cell was estimated using a physical disector with two adjacent ultrathin sections of 100-nm thickness mounted on one slot grid (32). The mean size of an individual lamellar body was then estimated by dividing total lamellar body volume by lamellar body number. In addition, the volume-weighted mean volume of lamellar bodies, a parameter that contains information on mean particle size as well as variation in size, was estimated by the point-sampled intercepts method (13). For the purpose of the present study, the total amount of lamellar bodies was defined as the intracellular surfactant pool.

**Real-time quantitativePCR.** Expression of MMP-12 was assessed by real-time quantitative PCR using four to seven mice per genotype. Bronchoalveolar lavage fluid was centrifuged at 250 g for 5 min at 4°C. RNA was extracted from the cell pellet and from nonlavaged lung using the RNeasy reagents according to the manufacturer’s recommendations (Qiagen, Alameda, CA). Total RNA (2 μg per sample) was reverse transcribed using RETROscript reagents (Ambion, Austin, TX), substituting random hexamers for random decamers. Real-time quantitative PCR amplification of total cDNA was performed using an ABI PRISM 7900HT Sequence Detector System with a 384 well block (Applied Biosystems, Foster City, CA). Reaction conditions were AmpliTaq Gold (Applied Biosystems) 0.25 μM with accompanying reaction buffer, 2.5 mM MgCl2, 100 μM each dNTP, 300 μM each primer, 0.05 × SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR), and 10 ng of cDNA in 10-μl reactions using a two-step PCR program: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. For MMP-12, the forward primer was 5′-GACTAAAAAGTTTTCAGGCACAAACC-3′, and the reverse primer was 5′-TTATTTGACTTGGATATTGGAATG-3′. Differences in cDNA input were corrected for by normalization to signals obtained using primers specific for GAPDH. For GAPDH, the forward primer was 5′-TGGCAAGAGAGCCCTATCC-3′, and the reverse primer was 5′-TGGCCCTCTGGTTATTAGG-3′. Standard curves for MMP-12 and GAPDH were constructed on each plate from serial log dilutions of a stock cDNA pool of several experimental samples (10 pg–100 ng), and relative quantification in triplicate for each experimental sample was obtained using the standard curve method. Control reactions were performed without reverse transcriptase and in the absence of target DNA.

**Immunohistochemistry.** Lungs were fixed by in situ tracheal instillation of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After removal, they were immersed in the same fixative at room temperature for 60–90 min. They were then cut into pieces and fixed further in fresh fixative at 4°C overnight in 4% paraformaldehyde and 0.1 M phosphate buffer with 30% sucrose. Tissue samples were then embedded in Tissue Tek OCT compound
Cryostat sections (3 µm) were mounted onto Fisherbrand Superfrost Plus slides and stored at −20°C. Thawed sections were treated for 10 min at 95°C in Target Retrieval Solution (Dako, Carpinteria, CA) and rinsed in PBS containing 0.1% BSA and 0.3% Triton X-100. The sections were incubated with this buffer plus 10% goat serum for 1 h at room temperature to block nonspecific binding. The tissue sections were incubated with specific anti-MMP-12 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10 at 4°C overnight. After washing with PBS containing 0.1% BSA and 0.3% Triton X-100, sections were treated with rabbit anti-goat IgG secondary antibody conjugated to Alexa 488 (Molecular Probes) overnight at 4°C. Sections were washed sequentially in PBS followed by double-distilled water. Coverslips were mounted with Prolong (Molecular Probes). The bound antibodies were detected under fluorescence microscopy and photographed using 35-mm Kodak Ektachrome 400 film. Control sections treated with secondary antibody alone were negative in all experiments.

Statistics. Stereological data were analyzed with the two-sided nonparametric Mann-Whitney’s U-test. Tests were performed using the Statistica 6.0 software (StatSoft, Hamburg, Germany). A value of P < 0.05 was considered significant.

RESULTS

Light and electron microscopy. In WT mice (Fig. 1A), parenchymal architecture looked normal. Alveoli had thin septae and normal-appearing type II cells. In contrast, D−G+ mice (Fig. 1B) showed apparently enlarged distal air spaces and accumulation of intra-alveolar surfactant material, organized as small and large lamellar body-like forms, tubular myelin, and unilamellar vesicles (B). Surfactant material also accumulates in the alveoli of D−G− mice. D+G− mice also appear to have an enlargement of distal air spaces (C). In D−G− mice, the accumulation of surfactant material within the alveoli as well as distal air space enlargement seems to be most intense (D).

Fig. 1. Low-power electron micrographs from wild-type (WT; A), D−G+ (surfactant protein D-deficient; B), D+G− (granulocyte/macrophage colony-stimulating factor-deficient; C), and D−G− (SP-D and GM-CSF double deficient; D) mice. Compared with WT (A), D−G+ mice show enlarged air spaces. Alveoli are filled with surfactant material organized as lamellar body-like forms, tubular myelin, and unilamellar vesicles (B). Surfactant material also accumulates in the alveoli of D−G− mice. D+G− mice also appear to have an enlargement of distal air spaces (C). In D−G− mice, the accumulation of surfactant material within the alveoli as well as distal air space enlargement seems to be most intense (D).
These findings are consistent with emphysema of a roughly similar degree in both D−/− and D+/+ mice. When compared with D−/− and D+/+ mice, the double-deficient D−/− mice showed even more pronounced emphysema, as indicated by lowest alveolar surface areas, thousand alveolar seps, lowest alveolar numbers, and largest alveolar sizes (Fig. 2). In all genotypes, emphysema was not reflected at the level of densities: the volume fractions of parenchyma tissue, air spaces, and septal tissue completely lacked information on these profound structural alterations.

D−/− mice showed an increase in both number and size of alveolar type II cells when compared with WT mice (Fig. 3). The total volume of lamellar bodies per cell and per lung was also decreased in D−/− mice (Fig. 4), with a slight, although not statistically significant, reduction in number per cell and a significant reduction in mean size (Fig. 5). Thus D−/− mice showed hyperplasia and hypertrophy of type II cells with an increased intracellular surfactant pool due to an increased number of lamellar bodies per cell, whereas D+/+ mice showed smaller type II cells with a decreased intracellular surfactant pool due to a decreased number and size of lamellar bodies. In D−/− mice, the type II cell alterations were partly normalized with a number and size between WT and D−/− mice (Fig. 3). Moreover, intracellular surfactant pool size, as indicated by the total volume of lamellar bodies per cell and per lung (Fig. 4) as well as the number and size of lamellar bodies (Fig. 5), was completely normalized in D−/− mice.

Compared with WT mice, alveolar macrophages showed an increase both in number and in size in D−/− mice (Fig. 6). In D+/+ mice, alveolar macrophage number was decreased, whereas their size was not statistically different from WT mice.
In D−G− mice, the number of alveolar macrophages per lung normalized to WT values, whereas their mean size remained increased in the same range as in D−G+ mice.

**Real-time quantitative PCR.** To demonstrate the anticipated effect of GM-CSF deficiency on alveolar macrophage function, real-time quantitative PCR and immunohistochemistry for MMP-12, shown to be regulated by GM-CSF (26, 43) and associated with emphysema development in other models (14, 29), were performed. Results for analysis of MMP-12 expression in whole lung tissue and alveolar macrophages recovered from bronchoalveolar lavage are shown in Fig. 7. Compared with WT, expression of MMP-12 was increased >300-fold in alveolar macrophages and ~30-fold in whole lung tissue in D−G+ mice. MMP-12 expression of D−G− mice was increased >70-fold in alveolar macrophages and ~3.5-fold in whole lung compared with WT, but decreased ~4.5-fold in alveolar macrophages and ~8.5-fold in whole lung compared with D−G+ mice.

**Immunohistochemistry.** Results for immunohistochemistry for MMP-12 are shown in Fig. 8. Immunostaining for MMP-12 was prominent and bright in tissue from D−G+ mice. In comparison, D−G− mice showed less staining.

**DISCUSSION**

Qualitatively, mice lacking SP-D develop a progressive, destructive air space enlargement associated with increased numbers of peribronchial-activated T lymphocytes and enlarged foamy alveolar macrophages (6, 15, 25, 41). Although the initiating stimulus of the chronic, noninfectious inflammation is unknown, the increased numbers of activated alveolar macrophages expressing high levels of MMP, particularly macrophage metalloelastase (MMP-12), ROS, and RNS are likely important to the progressive lung remodeling that occurs (2, 41, 45). The relative importance of particular cell types or specific mediators in this pathology has, however, not been established. GM-CSF is an important regulator of terminal alveolar macrophage differentiation and function. GM-CSF signaling is required for the expression of the transcription factor PU.1 that in turn regulates the expression of a large number of macrophage effector functions, including cell surface receptor expression, phagocytosis, ROS production, and surfactant degradation (3–5, 35). GM-CSF also regulates MMP expression, including positive regulation of MMP-12 (26, 43). This study was designed to test the hypothesis that GM-CSF-dependent type II cell and alveolar macrophage activation contributes to the hyperplasia and hypertrophy of type II cells and alveolar macrophages, respectively, and to the lung remodeling associated with SP-D deficiency. The heterogeneity of both the cellular and parenchymal changes required a quantitative handling of the lung histology to adequately test the hypothesis.

Until recently, the quantification of emphysematous changes in the lung was hampered by the fact that a design-based stereological method for the direct and unbiased estimation of alveolar number was missing. Such a method, based on the estimation of the Euler number of the network of alveolar openings, is now available (21, 31). The combined estimation of alveolar number and size as well as total alveolar surface area now allows for an unambiguous determination of loss of...
alveoli and loss of gas exchange surface in cases of destructive emphysema as opposed to simple air space enlargement, as indicated by an increase in mean linear intercept length, which has been used as the standard method in lung biology for many years. In addition, adaptations of the disector principle (37) for unbiased counting of alveolar type II cells (at the light microscopy level) and their lamellar bodies (at the electron microscopy level) have also become available (32).

Our stereological data quantitatively confirm changes in the structure of the parenchyma that are consistent with emphysema in SP-D-deficient mice. Specifically, these mice have decreased alveolar epithelial surface areas, thicker alveolar septae, and fewer but larger alveoli compared with WT controls. Surprisingly, these changes in structure are also present to an almost similar degree in GM-CSF-deficient mice and are significantly more, rather than less, pronounced in SP-D/GM-CSF double-deficient mice. As reported, these emphysema-like changes are not present in SP-D-deficient mice until the formation of alveoli is completed (41). The changes are therefore most likely the result of progressive destruction of parenchyma rather than an arrest of alveolarization. It remains to be shown for GM-CSF-deficient mice whether the emphysema-like pathology present in these mice is destructive in nature, too. Together, our results indicate that GM-CSF is not necessary for emphysema development in SP-D-deficient mice.

GM-CSF-deficient alveolar macrophages produce significantly less ROS in response to group B streptococcal infection (27), and MMP-12 expression is reduced 4.5-fold in alveolar macrophages from SP-D/GM-CSF double-deficient mice compared with alveolar macrophages from SP-D-deficient mice. The unexpected result of more pronounced emphysematous alterations in SP-D/GM-CSF double-deficient mice, therefore, suggests that products other than either ROS or MMP-12 derived from alveolar macrophages or other cell types mediate the emphysema-like pathology in this model. The idea that products from alveolar macrophages alone cannot be responsible for the full extent of emphysema in SP-D/GM-CSF double-deficient mice is supported by our stereological data showing that the increase in the number of alveolar macrophages in SP-D-deficient mice is fully normalized in SP-D/GM-CSF double-deficient mice. Lymphocytes, dendritic cells, alveolar type II cells, fibroblasts, and capillary endothelial cells (1, 24) might contribute significantly to the tissue remodeling in both SP-D-deficient and GM-CSF-deficient mice. Our results contrast with the apparently specific role of alveolar macrophage-derived MMP-12 in the emphysema produced by either cigarette smoke (14) or \( \beta_6 \)-integrin deficiency (29). Specific mediators of emphysema in SP-D-deficient mice are the subject of current investigation.

Recent work on the roles of GM-CSF in pulmonary homeostasis has focused on GM-CSF effects on alveolar macrophages since a defect in the GM-CSF-regulated degradation of surfactant by alveolar macrophages seems to be responsible for the alveolar proteinosis-like pathology in GM-CSF-deficient mice and for the disease in humans who have circulating neutralizing autoantibodies against GM-CSF (38, 39). How-

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Fig. 5: A: number of lamellar bodies per type II cell \([N_{lb,typeII}]\). B: size of lamellar bodies \([b_{lb}]\). D–G+ mice have more lamellar bodies per type II cell. In D+G– mice, lamellar bodies are smaller in size. D–G– mice have lamellar bodies that are normal in number and size.

Fig. 6: A: number of alveolar macrophages per lung \([N_{alvmac}]\). B: size of alveolar macrophages \([b_{alvmac}]\). D+G– mice have fewer alveolar macrophages. Alveolar macrophages in D–G– mice are normal in number but are still increased in size.
ever, GM-CSF might also act directly on alveolar epithelial type II cells since type II cells express both GM-CSF and a functional GM-CSF receptor (20, 38, 39). Mice overexpressing GM-CSF develop marked hyperplasia of alveolar type II cells (20). The present study shows that, in contrast to the emphysema, the type II cell alterations in number and size as well as the lamellar body alterations in SP-D-deficient mice are largely corrected in the absence of GM-CSF, indicating that GM-CSF is necessary for type II cell proliferation and hypertrophy in this model. Although not examined in our study, it is also possible that GM-CSF could mediate the expression and release of inflammatory mediators by type II cells. Interestingly, our data also demonstrate that the deletion of GM-CSF alone leads to a significant reduction in the size of type II cells as well as their lamellar bodies, thus resulting in a more than threefold decrease in the total volume of lamellar bodies per lung. This novel observation is in apparent contrast to data by Ikegami et al. (22), who found a sixfold increase in lung tissue saturated phosphatidylcholine pool size in GM-CSF-deficient mice. This discrepancy is most likely due to methodological differences leading to different definitions of the intracellular surfactant pool by morphological (lamellar bodies in type II cells) or biochemical (saturated phosphatidylcholine in lung tissue after alveolar lavage) criteria. Together, our findings support the concept that type II cell surfactant metabolism is, either directly or indirectly, significantly influenced by GM-CSF and suggest that GM-CSF might be involved in the regulation of lamellar body formation and/or maturation.

In conclusion, the present study has shown that the loss of GM-CSF in SP-D-deficient mice results in more pronounced emphysema but corrects the marked alterations of alveolar type II cell number and size and the intracellular surfactant pool. This suggests the type II cell and intracellular surfactant abnormalities in both SP-D-deficient and GM-CSF-deficient mice may not be causally related to the chronic inflammation and emphysema seen in both models. This concept is in line with recent findings in SP-D-deficient mice expressing an SP-D/conglutinin fusion protein (46) and SP-D-deficient mice.
in which SP-D was conditionally replaced (47). In both models, alterations in lipid metabolism were corrected, whereas emphysema, based on qualitative data, was not. These results suggest that distinct pathways and signaling mechanisms are involved in the complex lung alterations and altered surfactant homeostasis seen in SP-D-deficient mice.

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