Surfactant Dysfunction and Lung Inflammation in the Female Mouse Model of Lymphangioleiomyomatosis

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Abstract

Pulmonary lymphangioleiomyomatosis (LAM) is a rare lung disease caused by mutations of the tumor suppressor genes, *tuberous sclerosis complex (TSC) 1* or *TSC2*. LAM affects women almost exclusively, and it is characterized by neoplastic growth of atypical smooth muscle–like TSC2-null LAM cells in the pulmonary interstitium, cystic destruction of lung parenchyma, and progressive decline in lung function. In this study, we hypothesized that TSC2-null lesions promote a proinflammatory environment, which contributes to lung parenchyma destruction. Using a TSC2-null female murine LAM model, we demonstrate that TSC2-null lesions promote alveolar macrophage accumulation, recruitment of immature multinucleated cells, an increased induction of proinflammatory genes, nitric oxide (NO) synthase 2, IL-6, chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP1), chemokine (C-X-C motif) ligand 1 (CXCL1)/keratinocyte chemoattractant (KC), and up-regulation of IL-6, KC, MCP-1, and transforming growth factor- β 1 levels in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid also contained an increased level of surfactant protein (SP)-D, but not SP-A, significant reduction of SP-B levels, and a resultant increase in alveolar

surface tension. Consistent with the growth of TSC2-null lesions, NO levels were also increased and, in turn, modified SP-D through S-nitrosylation, forming S-nitrosylated SP-D, a known consequence of lung inflammation. Progressive growth of TSC2-null lesions was accompanied by elevated levels of matrix metalloproteinase-3 and -9. This report demonstrates a link between growth of TSC2-null lesions and inflammation-induced surfactant dysfunction that might contribute to lung destruction in LAM.

Keywords: interstitial lung disease; nitric oxide; surfactant protein-D; tuberous sclerosis complex 2; animal models

Clinical Relevance

This study demonstrates that tuberous sclerosis complex 2–null induced pulmonary inflammation, as defined by increased nitric oxide synthase 2 expression, S-nitrosylation of surfactant protein-D, and surfactants dysfunction, might contribute to airspace enlargement and respiratory impairment, key aspects of the pathology of human lymphangioleiomyomatosis.

Pulmonary lymphangioleiomyomatosis (LAM) is a rare, unrelenting parenchymal lung disease characterized by the proliferation of abnormal smooth muscle–like “LAM cells,” leading to cystic destruction of the lung parenchyma

and progressive respiratory failure (1, 2). LAM cells are low-grade, destructive, and metastasizing neoplasm (3). A key element to the progression of the disease is parenchymal destruction within the lung that can lead to

pneumothorax and respiratory failure. The mechanisms by which LAM cells induce lung tissue destruction are unknown.

Although LAM has not been defined as an inflammatory disease, the high

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expression of inflammatory chemokines in bronchoalveolar lavage (BAL) fluid and nodules from patients with LAM (4) suggested the involvement of inflammation in the pathogenesis of LAM. Importantly, airway inflammation was reported in 61% of patients with LAM (5). One-third of patients with LAM with airway obstruction respond to bronchodilators, such as albuterol, a β_2 -adrenoreceptor agonist, with an increase in FEV₁ of 12% and 200 ml above baseline values (5). We have reported increased levels of proinflammatory cytokines in a female mouse LAM model, suggesting that inflammation may cause the alveolar destruction (6). Published *in vitro* data also demonstrate that the tuberous sclerosis complex (TSC) 2-mammalian target of rapamycin (mTOR) pathway regulates inflammatory responses after bacterial stimulation in mononuclear phagocytes, suggesting that the TSC2-mTOR pathway is a regulator of innate immune homeostasis (7). However, the exact mechanisms of pulmonary inflammation and its contribution to lung destruction in LAM are not well understood.

In the lung, nitric oxide (NO) is a key regulator, controlling varied processes, including airway tone and inflammation (8, 9). In particular, up-regulation of NO production via the inducible isoform of NO synthase (iNOS; coded by NOS2 gene) has been shown to be important in innate immune responses (10, 11), in promoting macrophage activation (12), and in the development of emphysematous and interstitial lung pathologies (13). NO has also been demonstrated to have both pro- and antitumorogenic roles (14). However, whether NO has a role in the pathophysiology of LAM is unclear.

The lung lining fluid, acting as a surfactant, also contains key regulatory elements of the innate immune system. Produced predominantly by type II epithelial cells, there are four surfactant proteins (SPs), SP-A, -B, -C, and -D (15). Whereas SP-B and -C are critical to surface activity and lung function (16), SP-A and -D are collectins, which facilitate pathogen clearance and regulate immune function in the lung. Homozygous depletion of SP-D (*Sftpd*^{-/-}) results in an enhanced baseline inflammatory state and increased susceptibility to bacterial and viral lung infection (17–20).

Figure 1. Tuberous sclerosis complex (TSC) 2-null lung lesions induce proinflammatory cytokines and gene activation. (A) Representative micrographs of hematoxylin and eosin staining of lung sections from untreated control mice and mice with TSC2-null lesions. (B) Bronchoalveolar lavage (BAL) fluid was collected at Day 21 after TSC2-null cell injection and analyzed for cytokine level by the Aushon Searchlight Protein Array multiplex ELISA. Data are expressed as mean values $\pm$ SEM; $n = 10$ /group. * $P < 0.05$ versus untreated control mice by t test. (C) RNA was extracted from BAL cells isolated from lungs of untreated control mice and mice with TSC2-null lesions. Gene markers were quantified by quantitative RT-PCR, as described in the MATERIALS AND METHODS. Threshold cycle (Ct) values obtained were normalized to β -actin signals and further analyzed using the relative quantization ($\Delta\Delta C_t$) method. Data are expressed as fold change (means $\pm$ SEM; $n = 5$ –8 in each group). Data are expressed as mean values $\pm$ SEM; $n = 10$ /group. * $P < 0.05$ versus untreated control mice by t test. CCL2, chemokine (C-C motif) ligand 2; CXCL1, chemokine (C-X-C motif) ligand 1; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; mRNA, messenger RNA; TGF- β 1, transforming growth factor β 1.

Furthermore, the chronic inflammation seen in the *Sftpd*^{-/-} mice is associated with activation of NOS2 gene expression (10, 19) and parenchymal tissue destruction (13). Importantly, pharmacological inhibition of iNOS or ablation of the NOS2 gene within *Sftpd*^{-/-} mice results in reduction of inflammation (13, 21). We have identified NO-mediated modification of SP-D in a variety of human diseases, including asthma and Hermansky-Pudlak syndrome (22, 23). The link between SPs, their function, and lung destruction in LAM have not been investigated.

In this study, using a female murine LAM model, we demonstrate that TSC2-null lesions activate the innate immune response, resulting in inflammation within the lung lining, impaired SP-B production and surfactant function, activated NOS2 gene expression, NO production, and modification of SP-D. We propose that these events might contribute to lung tissue destruction in LAM.

Materials and Methods

Female Murine LAM Model

The female TSC2-null murine LAM model, previously described and characterized (6), validates metastatic cell dissemination in LAM (3). Briefly, 8-week-old, athymic nude female mice (NCRNU-F; Taconic) were injected with (10⁶) TSC2-null cells in PBS into the tail vein. Mice, injected with PBS only, were used as untreated controls. Animals were observed three times per week for signs of pulmonary distress and weight loss. Body weight progressively decreased starting on Day 10, but was not greater than 20% below the initial body weight. At 3 weeks after injection, progressive growth of TSC2-null lesions induced marked airspace enlargement in the lung (Figure 1), in concordance with previous work (6). After 21 days, animals were killed, followed by BAL and tissue collection, and analysis. All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania (Philadelphia, PA).

BALF Analyses

At 21 days after injection, mice were killed, and lungs were lavaged with 0.5- to 1-ml aliquots of sterile 0.9% saline to a total of

5 ml, as previously described (6). Recovered BAL fluids were centrifuged at 400 × g for 10 minutes at 4°C and cell pellets were resuspended in PBS, followed by cell count using a Z1 Counter particle counter (Beckman-Coulter, Inc., Miami, FL). Differential cell counts were performed manually on cytopreparations stained with Diff-Quik. Aliquots of the cell-free BAL fluid were analyzed for cytokine levels by Multiplex (Aushon, Billerica, MA) and NO metabolites by chemical reduction and chemiluminescence using the Ionics/Sievers Nitric Oxide Analyzer 280 (NOA 280; Ionics Instruments, Boulder, CO) (24).

Surfactant Fractionation and Analyses

The cell-free BAL fluids were centrifuged at 20,000 × g for 40 minutes at 4°C for separation into two surfactant fractions: the biophysically active large-aggregate (LA) form, and the biophysically inactive small-aggregate (SA) form. The resulting LA pellets were resuspended in saline. Total protein and phospholipid contents within LA and SA surfactant fractions were determined by the bicinchoninic acid method (Pierce, Rockford, IL) and by the method of Bartlett, respectively, as previously described (23). The biophysical activity of recovered LA surfactant was measured in a Capillary Surfactometer (Calmia Medical, Canada) and has been extensively described previously (24, 25). Samples of LA fractions of BAL fluid were diluted with saline to a total phospholipids concentration of 0.5–1.0 mg/ml, and 0.5 µl was deposited into the narrow section of the glass capillary. Data were expressed as percentage of capillary openness, where 100% fully open capillary corresponds to minimum surface tension. Each sample was analyzed in triplicate.

Western Blotting

Detection of SP-B level in LA fraction, SP-A and SP-D levels, and its multimeric structure in BAL was performed as previously described (24, 26, 27) using a polyclonal anti-SP-B (Abcam, Cambridge, MA) or polyclonal in-house anti-SP-D and anti-SP-A antibodies. Detection of S-nitrosylated SP-D (SNO-SP-D) in BAL fluid was performed via the biotin switch method, as previously described (11, 23).

Flow Cytometric Analysis

Cells collected in BAL fluid of either untreated control mice or mice with TSC2-null lesions were immunostained for flow cytometry using the following fluoro-chrome-labeled monoclonal antibodies according to manufacturers' protocol: FITC-Cd11b, PE-Ly6C, PE/Cy7- F4/80, AF647-Ly6G (Biolegend, San Diego, CA) and Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA). Cells were incubated with TruStain FcX (anti-mouse CD16/32) (Biolegend) before antibody incubation. Single stains were used for gating purposes and in accordance with previous measurements using nonimmune IgG controls. Data were acquired with Gallios Flow Cytometer (Beckman-Coulter, Inc.) and analyzed with Kaluza software (Beckman-Coulter, Inc.).

Quantitative RT-PCR

The cells recovered from BAL fluid were analyzed for messenger RNA (mRNA) expression levels by quantitative RT-PCR according to the manufacturer's instructions (Applied Biosystems) as previously described (13). Obtained threshold cycle (Ct) values were normalized to β-actin signals and further analyzed using the relative quantization (ΔΔCt) method.

Statistical Analysis

Data are shown as mean (±SEM). Statistically significant differences between groups of mice were determined by Student's *t* test. Values of *P* less than 0.05 were considered significant.

Results

TSC2-Null Lung Lesions Induce Proinflammatory Cytokines and Genes Activation

We previously established and characterized the mouse LAM model, which manifests by progressive growth of TSC2-null lesions in the lung (6). This experimental LAM model demonstrates the metastatic potential of TSC2-null cells and supports its use as a model for LAM (3). Consistent with our previous observations, the lungs with TSC2-null lesions (Figure 1A, *upper panels*) contain multiple emphysematous-like areas, displaying enlarged airspaces, heterogeneously distributed, and interspersed with interstitial infiltrates

comprised predominantly of mononuclear inflammatory cells (Figure 1A, lower panels).

To determine whether TSC2-null lung lesions induce inflammatory cytokine release, a profile of relevant cytokines/chemokines were measured in the BAL fluid from both experimental groups (mice with TSC2-null lesions and littermate untreated controls). The concentration of cytokines, IL-6, chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1), chemokine (C-X-C

motif) ligand 1 (CXCL1)/keratinocyte chemoattractant (KC), matrix metalloproteinase (MMP) 3, MMP9, and transforming growth factor (TGF)- β 1, were markedly increased in BAL from lungs with TSC2-null lesions when compared with untreated control lungs (Figure 1B). The levels of granulocyte/macrophage colony-stimulating factor, IFN- γ , IL-13, and eotaxin were not altered by the development of TSC2-null lesions (data not shown). The increased levels of IL-6,

CCL2/MCP-1, and CXCL1/KC suggested classic proinflammatory activation of macrophages. In accordance with these observations, examination of BAL cell mRNA reveals proinflammatory gene expression, IL-6, CXCL1/KC, and CCL2/MCP-1, without increases in alternative activation genes, IL-1 β and Ym1 (Figure 1C). The increase in metalloproteinase expression (MMP3 and MMP9) is indicative of inflammation-mediated tissue destruction (6).

Figure 2. TSC2-null lung lesions promote recruitment of immature myeloid cells. (A) Representative micrographs of cells recovered from BAL untreated control mice and mice with TSC2-null lesions. The red arrows indicate precursor cells, and the arrowheads denote multinucleated cells; magnification, $\times 20$. (B) Total BAL cells were determined by Coulter counting. (C) Differential cell counts were done using Diff-Quick staining of cytospin slides. Cells were identified as macrophages, eosinophils, multinucleated, or precursors by standard morphology. Values are shown as means $\pm$ SEM. $*P < 0.05$ versus untreated control mice by *t* test; $n = 10$ /group. (D) Representative micrographs of BAL cell distribution by size. (E) Representative flow cytogram of BAL cells stained for CD11b, F4/80, Ly6C, and Ly6G markers. Colors are drawn by population and quadrants by single-stain gating strategy. Upper panels show the CD11b and F4/80 expression of viable cells. CD11b $^+$ /F4/80 $^+$ cells are green in color, whereas purple color represents CD11b $^-$ /F4/80 $^+$, and black is negative for both. Lower panels show the Ly6C and Ly6G expression of cells gated on CD11b $^+$ /F4/80 $^+$. Flow cytograms are representative of five mice in each group. EP, eosinophils; MP, macrophages.

Interestingly, the profibrotic cytokine, TGF- β 1, which can suppress IFN- γ expression (28), was also increased in the lung lining fluid (Figure 1B).

TSC2-Null Lung Lesions Promote Recruitment of Immature Myeloid Cells

The expression of proinflammatory cytokines seen in Figure 1 is generally associated with the recruitment and classical activation of macrophages (12, 13). The BAL cells from mice with TSC2-null lesions and littermate untreated controls were visualized, counted, and analyzed by flow cytometry for surface marker expression. TSC2-null lesions were accompanied by increased total BAL cellularity, which consisted primarily of increased numbers of macrophages (Figures 2B–2D). Morphological differentiation (Figures 2A and 2C) revealed that BAL cells from untreated control lungs contained predominantly macrophages, whereas cells from lungs with TSC2-null lesions were heterogeneous in appearance and contained a variety of immune cells, including macrophages, multinucleated cells, and a large number of smaller cells (Figures 2A and 2D). These smaller cells had band- or ring-shaped nuclei with a higher nucleus-to-cytoplasm ratio than normal alveolar macrophages, features that are consistent with being myeloid progenitors (Figure 2A). To further characterize the cells of the lung lining, we examined surface marker expression by flow cytometry. As seen in Figure 2E (*top panels*), the predominance of cells in the BAL were positive for F4/80, a macrophage marker. A significant

portion of cells from mice with TSC2-null lesions, but not from untreated control mice, also expressed CD11b, an integrin that is associated with cellular movement and is an indicator of immaturity. To further characterize their phenotype, the F4/80⁺/CD11b⁺ cells were further analyzed for expression of the activation markers, Ly6G and Ly6C. As seen in Figure 2E (*lower panels*), these cells were indeterminate in their expression of Ly6C, but there was a clear subpopulation that was positive for Ly6G. These cells represent immature activated myeloid cells that have previously been shown to have immunosuppressive function (29).

TSC2-Null Lung Lesions Induce Dysfunction of Surfactant and Its Activity

Pulmonary inflammation has been shown to alter surfactant component homeostasis and function in both human and animal models (23, 26, 30–33). We examined the total protein and phospholipid contents in the LA and SA surfactant fractions of the BAL from untreated control mice and those with TSC2-null lesions. Table 1 shows that the total protein level in the LA and SA surfactant fractions were proportionately increased (3.7- and 3.3-fold, respectively) in mice with TSC2-null lesions, suggesting an increase in vascular permeability within these lungs. The phospholipid content of the LA fraction was increased by 2.6-fold in mice with TSC2-null lesions (Table 1), whereas the phospholipid level in the SA fraction was unaffected. Measurement of the LA for SP-B content, normalized to total phospholipid, shows that the presence of TSC2-null lesions reduces its expression

(Figure 3A). Because SP-B modulates surfactant biophysical activity, we directly determined whether mice with TSC2-null lesions exhibited surfactant dysfunction via capillary surfactometry (24, 25). LA surfactant from lungs with TSC2-lesions produced 45% less capillary opening time when compared with untreated control lungs, indicating a significant reduction in surfactant function. Thus, despite an increase in total phospholipid, the reduction in SP-B, and possibly the increase in vascular leak, as shown by the increased total protein, promoted an increase in the minimum surface tension. A loss of surface-active function within the lung lining would contribute to respiratory impairment as is seen in LAM.

In addition to the surface-active components, type II cells also produce the pulmonary collectins, SP-A and SP-D. To assess whether the reduction of SP-B content in the lungs with TSC2-null lesions was specific and not simply due to global type II cell impairment or dropout, BAL fluids from untreated control mice and mice with TSC2-null lesions were also analyzed for SP-A and SP-D. Total BAL SP-D level was significantly increased in mice with TSC2-null lesions compared with untreated control mice (Figure 3C). In contrast to differential changes in SP-B and SP-D, the SP-A protein content was not significantly altered by the presence of TSC2-null lesions (Figure 3D).

TSC2-Null Lung Lesions Are Associated with Enhanced Pulmonary Nitrogen Oxides Production

Previously, we showed that NOS2 induction and the production of nitrogen oxides accompanies both chronic and acute pulmonary inflammation in a variety of models (11, 22, 24, 34). In addition, NO appears to regulate mTORC1 function and autophagy within breast cancer cells (14). In this context, it is significant that the nitrogen oxide content of BAL fluid, and NOS2 mRNA expression in the BAL cells, were significantly increased in mice with TSC2-null lesions when compared with untreated control (Figures 4A and 4B). Importantly, NOS2 expression was 16-fold higher than ARG1 arginase 1 expression in mice with TSC2-null lesions when compared with untreated control (Figure 4B), confirming a bias toward NO production by pulmonary inflammatory cells within this model of LAM.

Table 1. Tuberous Sclerosis Complex 2–Null Lung Lesions Alter Surfactant Homeostasis

	Protein (μ g)		Phospholipids (μ g)	
	LA	SA	LA	SA
Control	25 $\pm$ 4	310 $\pm$ 7	78 $\pm$ 13	42 $\pm$ 10
TSC2-null	92 $\pm$ 5*	1,014 $\pm$ 99*	206 $\pm$ 29*	67 $\pm$ 13

Definition of abbreviations: LA, large aggregate; SA, small aggregate; TSC2, tuberous sclerosis complex 2.

LA and SA fractions from bronchoalveolar lavage fluid of untreated control lungs and lungs with TSC2-null lesions were analyzed by the Bartlett method for phospholipids and by bichinchoninic acid assay for protein contents. Data shown are mean $\pm$ SE.

* $P < 0.05$ versus untreated control lungs.

Figure 3. TSC2-null lung lesions induce dysfunction of surfactant and its activity. (A) Equal amount of total phospholipids from large-aggregate (LA) fractions of untreated control mice and mice with TSC2-null lesions were subjected to SDS-PAGE under reduced conditions, followed by immunoblotting with anti-surfactant protein (SP)-B antibody. *Upper panel:* representative micrographs of SP-B content. *Lower panel:* densitometric quantification of SP-B content. Mean values of all the samples from untreated control ($n = 10$) and TSC2-null ($n = 10$) groups were calculated and presented as a percentage of untreated control mice. Values are shown as means $\pm$ SEM. * $P < 0.05$ versus untreated control mice by *t* test. (B) Minimum of surface tension was determined by measuring capillary openness by capillary surfactometer, as described in MATERIALS AND METHODS. Values are shown as a percentage of capillary openness, where 100% is fully open capillary that corresponds to the minimum surface tension (values are obtained by averaging triplicate measurements of each sample; mean $\pm$ SEM; $n = 10$ /group); * $P < 0.05$ versus untreated control mice by *t* test. (C and D) BAL fluid was collected at 21 days after TSC2-null cell injection and analyzed for SP-D (C) or SP-A (D) protein content. *Upper panels:* representative micrographs of total BAL fluid SP-A or SP-D content. BAL samples containing equal volume were subjected to SDS-PAGE under reduced conditions, followed by immunoblotting with anti-SP-A or SP-D antibody. *Lower panels:* densitometric quantification of SP-A and SP-D content presented as a percentage of untreated control mice. Values are shown as means $\pm$ SEM; $n = 6$ /group.

TSC2-Null Lesions Induce S-Nitrosylation of SP-D

We have previously shown that SP-D is susceptible to post-translational modification by NO via S-nitrosylation (SNO-SP-D) and can serve as a biomarker of pulmonary inflammation (35). Therefore, we examined SNO-SP-D levels in BAL fluids of both untreated control and mice with TSC2-null lesions

(Figure 5). BAL fluids were normalized for equal total SP-D loading (Figure 5C). There was a significant increase in SNO-SP-D content in mice with TSC2-null lesions (Figure 5A). In agreement with increased level of SNO-SP-D, there was observed disruption of SP-D multimeric structure in the BAL of mice with TSC2-null lesions, as shown by the presence of lower molecular weight bands on native electrophoresis

(Figure 5B). These lower molecular weight forms were not observed in untreated control mouse BAL. Previously, we have observed that SNO-SP-D is an activator of macrophage function and that it is associated with a variety of pulmonary inflammatory diseases (11, 22, 23, 36), indicating that NO-mediated modification of SP-D may play a role in the tissue destruction observed in this model of LAM.

Discussion

LAM is a progressive debilitating disease in which neoplastic lesion growth is associated with proliferation of the lymph vessels. However, the disease is also characterized by lung tissue destruction and loss of septation, as has been seen in a variety of pulmonary inflammatory diseases, such as emphysema. Lung inflammation and SPs are key components in dysregulated lung homeostasis, causing emphysematous alveolar changes. Little is known about SPs and their regulation in pulmonary LAM. In this study, using a mouse LAM model, we demonstrated that growth of TSC2-null lung lesions promoted recruitment of activated inflammatory cells, proinflammatory cytokine influx, and surfactant dysfunction.

Chronic inflammation is highly correlated with many types of human cancer, where both tumor cells and stromal cells elaborate chemokines and cytokines (12). In this study, we demonstrated that CCL2/MCP-1 and CXCL1/KC chemokine levels were higher in BAL fluid from lungs with TSC2-null lesions than from untreated control lungs. Moreover, the increase in CCL2/MCP1 and CXCL1/KC chemokine levels is associated with the recruitment of macrophages and neutrophils into the lung lining. This observation is in agreement with recently published data that CCL2/MCP-1 level in BAL fluid from patients with LAM was higher when compared with healthy volunteers (4). It has been recently reported that CCL2/MCP-1 selectively attracts cells with dysfunctional TSC2 and is associated with LAM nodules in roughly 70% of patients, suggesting that this chemokine could be involved in the recruitment of LAM cells to the lung (36).

In addition, the observed increase in TGF- β 1 level is consistent with tumorigenesis in the lungs, and may be

Figure 4. TSC2-null lung lesions enhance pulmonary nitrogen oxides production. (A) BAL fluid was collected at 21 days after TSC2-null cell injection and analyzed for NO metabolites by chemical reduction, as described in MATERIALS AND METHODS. Results are expressed as means + SEM; * $P < 0.05$ versus untreated control mice by t test; $n = 5$ for untreated control group; $n = 12$ for TSC2-null group. (B) Cells recovered from BAL fluid were analyzed by quantitative RT-PCR for nitric oxide synthase (NOS) 2 and arginase 1 (ARG) gene expression and normalized to β -actin. All samples were run in triplicate; $n = 5$ for untreated control group; $n = 12$ for TSC2-null group.

mechanistically related to the extensive formation and growth of TSC2-null lesions. Examination of the inflammatory cells released to the lung lining reveals a large number of macrophages and recruited multinucleated cells, which are morphologically similar to tumor-associated neutrophils. These observations are indicative of a “left shift” to the young, less well differentiated neutrophils

and neutrophil-precursor cells (38, 39). Appearance of neutrophil-precursor cells generally reflects early release of myeloid cells from the bone marrow, due to acute inflammation.

It is likely that distinct differentiation programs of inflammatory cells occur in different states depending on the cytokine milieu. It has been reported that, in the tumor microenvironment, tumor-

associated neutrophils and immature myeloid cells support tumor growth by producing angiogenic factors and matrix-degrading enzymes (40, 41), and suppress the antitumor immune response (42). In this study, we found that immature myeloid cells are recruited to the lining of lungs with TSC2-null lesions. Such immature cells could contribute to tumor growth through the inhibition of T-cell function, although in a nude mouse model, this is unlikely to be a significant contribution. The presence of these cells in the lung lining fluid, rather than within the tumor, has implications for the initiation of tissue destruction observed in this model, and warrants further investigation in an immunocompetent mouse model.

Inflammatory cells can release various proteases, leading to destruction of the extracellular matrix and subsequent loss of the alveolar units (43). Pulmonary inflammation contributes to respiratory impairment, at least in part, by disrupting the pulmonary surfactant system (24, 44). Pulmonary surfactant is a surface-active mixture of phospholipids and hydrophobic proteins, SP-B and SP-C, secreted by alveolar epithelial type II cells to reduce surface tension at the air-liquid interface to maintain alveolar stability. Our study demonstrates that TSC2-null lesions induced down-regulation of SP-B, which was reflected by an increase in minimum surface tension achievable by the BAL *in vitro*. Because SP-B operates to increase phospholipid incorporation into the surfactant monolayer, it is critical to the reduction of surface tension within the alveoli. Thus, the decreased SP-B level in the lungs with TSC2-null lesions, along with the emphysematous alterations observed in these lungs (6), may lead to alveolar collapse, increased work of breathing, and impaired gas exchange. These data are consistent with the report by Taveira-DaSilva and coworkers (5) that the histological severity of the disease in patients with LAM correlates with the degree of impairment in gas exchange.

We have recently shown that SP-D, a pulmonary collectin and innate host defense protein with well-established immunomodulatory properties (11), can serve as a marker of pulmonary

Figure 5. TSC2-null lesions induce S-nitrosylation of SP-D. BAL fluid was collected at 21 days after TSC2-null cell injection and analyzed for SP-D protein content. (A) Representative micrographs of S-nitrosylated SP-D (SNO-SP-D) content. BAL samples containing equal SP-D loading were subjected to Biotin-Switch assay to determine amount of SNO-SP-D. (B) Representative micrographs of native SP-D. BAL samples containing equal SP-D loading were subjected to native gel electrophoresis to determine multimeric structure of SP-D. (C) Representative micrographs of equal SP-D loading. The net intensity of the samples from Figure 3C (upper panel) was calculated for equal SP-D loading.

inflammation (35, 45). We have also demonstrated that, under inflammatory conditions, NO modifies SP-D through S-nitrosylation, resulting in disassembling of multimeric SP-D structure and initiates a proinflammatory response through NF- κ B activation (11). To determine whether TSC2-null lesions induce S-nitrosylation of SP-D, we examined BAL fluids for the presence of SP-D modifications. When controlled for input of SP-D, BAL of the lungs with TSC2-null lesions exhibited a significant increase in SNO-SP-D. Increased SNO-SP-D levels were also associated with the presence of lower molecular weight forms of SP-D, evident by native gel electrophoresis of the BAL fluids, consistent with our previous results in other models of inflammation (11, 22, 24). SP-D multimeric structure was not disrupted within the lungs of untreated control mice, as shown by native gel electrophoresis. Therefore, the balance between multimeric SP-D and trimeric SNO-SP-D forms in the lung with TSC2-null lesions was shifted to favor of trimeric SP-D forms. The increase in SNO-SP-D, as well as in low molecular weight forms of the protein, was associated

with increased NO production and NOS2 activity. These results indicate activation of inflammatory processes within lungs with TSC2-null lesions, although we do not know whether SNO-SP-D is cause or effect in this process.

A major limitation in the development of new strategies for LAM treatment has been the lack of a representative animal model (6, 46). In this study, we have further characterized the TSC2-null mouse LAM model established in nude mice to examine the role of inflammation in the pathology. Although an animal model cannot exactly reproduce the pathology and pathophysiology of human LAM, this study has allowed us to make several important observations, namely, that there is significant inflammatory activation, recruitment of activated myeloid cells, disruption of surfactant function, and increased iNOS-related signaling. Bearing in mind that there is inflammatory activation within this model, there is a significant limitation in the use of a nude mouse, namely, a lack of normal T lymphocyte function. T cells are an important aspect of the inflammatory response and play a key role in tumor

suppression. Therefore, our future goal is to develop the LAM model into an immunocompetent mouse to understand how T cells may alter this inflammatory activation within LAM and to determine whether the observed lung inflammatory response is specific to TSC2-null lesions. Understanding the role of each type of immune cell, and the relevant signaling pathways involved in LAM initiation and progression, is critical to the discovery of biomarkers specifically targeting cancer inflammation.

Our data using a female murine LAM model have established a link between growth of TSC2-null lesions in the lung, NOS2 activation, and inflammation-induced surfactant alterations. These studies suggest that TSC2-null-induced pulmonary inflammation might directly contribute to increased NOS2 expression, SNO-SP-D levels, and dysfunction of pulmonary surfactant, which, in turn, results in airspace enlargement and respiratory impairment, key aspects of the pathology of human LAM. ■

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

References

- Johnson SR. Lymphangioleiomyomatosis. *Eur Respir J* 2006;27:1056–1065.
- Taveira-DaSilva AM, Moss J. Lymphangioleiomyomatosis. *Cancer Control* 2006;13:276–285.
- Henske EP, McCormack FX. Lymphangioleiomyomatosis—a wolf in sheep’s clothing. *J Clin Invest* 2012;122:3807–3816.
- Pacheco-Rodriguez G, Steagall WK, Moss J. Chemokine-enhanced chemotaxis of lymphangioleiomyomatosis cells with mutations in the tumor suppressor TSC2 gene. *J Immunol* 2009;182:1270–1277.
- Taveira-DaSilva AM, Hedin C, Stylianou MP, Travis WD, Matsui K, Ferrans VJ, Moss J. Reversible airflow obstruction, proliferation of abnormal smooth muscle cells, and impairment of gas exchange as predictors of outcome of lymphangioleiomyomatosis. *Am J Respir Crit Care Med* 2001;164:1072–1076.
- Goncharova EA, Goncharov DA, Fehrenbach M, Khavin I, Ducka B, Hino O, Colby TV, Merrilees MJ, Haczku A, Albelda SM, et al. Prevention of alveolar destruction and airspace enlargement in a mouse model of pulmonary lymphangioleiomyomatosis (LAM). *Sci Transl Med* 2012;4:154ra134.
- Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, Kolbe T, Stulnig TM, Hörl WH, Hengstschräger M, et al. The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* 2008;29:565–577.
- Gow AJ. The biological chemistry of nitric oxide as it pertains to the extrapulmonary effects of inhaled nitric oxide. *Proc Am Thorac Soc* 2006;3:150–152.
- Foster MW, Hess DT, Stamler JS. Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol Med* 2009;15:391–404.
- Atochina EN, Beers MF, Hawgood S, Poulain F, Davis C, Fusaro T, Gow AJ. Surfactant protein-D, a mediator of innate lung immunity, alters the products of nitric oxide metabolism. *Am J Respir Cell Mol Biol* 2004;30:271–279.
- Guo CJ, Atochina-Vasserman EN, Abramova E, Foley JP, Zaman A, Crouch E, Beers MF, Savani RC, Gow AJ. S-nitrosylation of surfactant protein-D controls inflammatory function. *PLoS Biol* 2008;6:e266.
- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008;454:436–444.
- Knudsen L, Atochina-Vasserman EN, Guo CJ, Scott PA, Haenni B, Beers MF, Ochs M, Gow AJ. NOS2 is critical to the development of emphysema in Sftpd deficient mice but does not affect surfactant homeostasis. *PLoS One* 2014;9:e85722.
- Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, Wogan GN. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. *Proc Natl Acad Sci USA* 2013;110:E2950–E2957.
- Wright JR, Dobbs LG. Regulation of pulmonary surfactant secretion and clearance. *Annu Rev Physiol* 1991;53:395–414.
- Serrano AG, Perez-Gil J. Protein-lipid interactions and surface activity in the pulmonary surfactant system. *Chem Phys Lipids* 2006;141:105–118.
- LeVine AM, Whitsett JA, Hartshorn KL, Crouch EC, Korfhagen TR. Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. *J Immunol* 2001;167:5868–5873.
- Schaub B, Westlake RM, He H, Arestides R, Haley KJ, Campo M, Velasco G, Bellou A, Hawgood S, Poulain FR, et al. Surfactant protein D deficiency influences allergic immune responses. *Clin Exp Allergy* 2004;34:1819–1826.
- Atochina EN, Gow AJ, Beck JM, Haczku A, Inch A, Kadire H, Tomer Y, Davis C, Preston AM, Poulain F, et al. Delayed clearance of *Pneumocystis carinii* infection, increased inflammation, and altered nitric oxide metabolism in lungs of surfactant protein-D knockout mice. *J Infect Dis* 2004;189:1528–1539.
- Madan T, Reid KB, Clark H, Singh M, Nayak A, Sarma PU, Hawgood S, Kishore U. Susceptibility of mice genetically deficient in SP-A or SP-D gene to invasive pulmonary aspergillosis. *Mol Immunol* 2010;47:1923–1930.

21. Atochina-Vasserman EN, Beers MF, Kadire H, Tomer Y, Inch A, Scott P, Guo CJ, Gow AJ. Selective inhibition of inducible NO synthase activity *in vivo* reverses inflammatory abnormalities in surfactant protein D-deficient mice. *J Immunol* 2007;179:8090–8097.
22. Atochina-Vasserman EN, Winkler C, Abramova H, Schaumann F, Krug N, Gow AJ, Beers MF, Hohlfeld JM. Segmental allergen challenge alters multimeric structure and function of surfactant protein D in humans. *Am J Respir Crit Care Med* 2011;183:856–864.
23. Atochina-Vasserman EN, Bates SR, Zhang P, Abramova H, Zhang Z, Gonzales L, Tao JQ, Gochuico BR, Gahl W, Guo CJ, *et al.* Early alveolar epithelial dysfunction promotes lung inflammation in a mouse model of Hermansky-Pudlak syndrome. *Am J Respir Crit Care Med* 2011;184:449–458.
24. Atochina-Vasserman EN, Gow AJ, Abramova H, Guo CJ, Tomer Y, Preston AM, Beck JM, Beers MF. Immune reconstitution during pneumocystis lung infection: disruption of surfactant component expression and function by S-nitrosylation. *J Immunol* 2009;182:2277–2287.
25. Enhörning G. Pulmonary surfactant function studied with the pulsating bubble surfactometer (PBS) and the capillary surfactometer (CS). *Comp Biochem Physiol A Mol Integr Physiol* 2001;129:221–226.
26. Guttentag SH, Akhtar A, Tao JQ, Atochina E, Rusiniak ME, Swank RT, Bates SR. Defective surfactant secretion in a mouse model of Hermansky-Pudlak syndrome. *Am J Respir Cell Mol Biol* 2005;33:14–21.
27. Atochina EN, Beers MF, Scanlon ST, Preston AM, Beck JM. *P. carinii* induces selective alterations in component expression and biophysical activity of lung surfactant. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L599–L609.
28. Letterio JJ, Roberts AB. Regulation of immune responses by TGF- β . *Annu Rev Immunol* 1998;16:137–161.
29. Tam JW, Kullas AL, Mena P, Bliska JB, van der Velden AW. CD11b+ Ly6Chi Ly6G– immature myeloid cells recruited in response to *Salmonella enterica* serovar typhimurium infection exhibit protective and immunosuppressive properties. *Infect Immun* 2014;82:2606–2614.
30. Günther A, Ruppert C, Schmidt R, Markart P, Grimminger F, Walmrath D, Seeger W. Surfactant alteration and replacement in acute respiratory distress syndrome. *Respir Res* 2001;2:353–364.
31. Atochina EN, Beck JM, Scanlon ST, Preston AM, Beers MF. *Pneumocystis carinii* pneumonia alters expression and distribution of lung collectins SP-A and SP-D. *J Lab Clin Med* 2001;137:429–439.
32. Casey J, Kaplan J, Atochina-Vasserman EN, Gow AJ, Kadire H, Tomer Y, Fisher JH, Hawgood S, Savani RC, Beers MF. Alveolar surfactant protein D content modulates bleomycin-induced lung injury. *Am J Respir Crit Care Med* 2005;172:869–877.
33. Glasser SW, Senft AP, Maxfield MD, Ruetschilling TL, Baatz JE, Page K, Korfhagen TR. Genetic replacement of surfactant protein-C reduces respiratory syncytial virus induced lung injury. *Respir Res* 2013;14:19.
34. Shi JD, Golden T, Guo CJ, Tu SP, Scott P, Lee MJ, Yang CS, Gow AJ. Tocopherol supplementation reduces NO production and pulmonary inflammatory response to bleomycin. *Nitric Oxide* 2013;34:27–36.
35. Atochina-Vasserman EN. S-nitrosylation of surfactant protein D as a modulator of pulmonary inflammation. *Biochim Biophys Acta* 2012;1820:763–769.
36. Atochina-Vasserman EN, Beers MF, Gow AJ. Review: chemical and structural modifications of pulmonary collectins and their functional consequences. *Innate Immun* 2010;16:175–182.
37. Pacheco-Rodriguez G, Moss J. The role of chemokines in migration of metastatic-like lymphangioleiomyomatosis cells. *Crit Rev Immunol* 2010;30:387–394.
38. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF- β : “N1” versus “N2” TAN. *Cancer Cell* 2009;16:183–194.
39. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002;23:549–555.
40. Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. *J Exp Med* 1995;181:435–440.
41. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci USA* 2008;105:2640–2645.
42. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res* 2001;61:4756–4760.
43. Mouded M, Egea EE, Brown MJ, Hanlon SM, Houghton AM, Tsai LW, Ingenito EP, Shapiro SD. Epithelial cell apoptosis causes acute lung injury masquerading as emphysema. *Am J Respir Cell Mol Biol* 2009;41:407–414.
44. Wright K, Kolios G, Westwick J, Ward SG. Cytokine-induced apoptosis in epithelial HT-29 cells is independent of nitric oxide formation: evidence for an interleukin-13-driven phosphatidylinositol 3-kinase-dependent survival mechanism. *J Biol Chem* 1999;274:17193–17201.
45. Matalon S, Shrestha K, Kirk M, Waldheuser S, McDonald B, Smith K, Gao Z, Belaaouaj A, Crouch EC. Modification of surfactant protein D by reactive oxygen–nitrogen intermediates is accompanied by loss of aggregating activity, *in vitro* and *in vivo*. *FASEB J* 2009;23:1415–1430.
46. Kwiatkowski DJ. Animal models of LAM and TSC. *Lymphat Res Biol* 2010;8:51–57.