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Identification of an autoantigen demonstrates a link between interstitial lung disease and a defect in central tolerance

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Abstract

Interstitial lung disease (ILD) is a common manifestation of systemic autoimmunity characterized by progressive inflammation or scarring of the lungs. Patients who develop these complications can exhibit significantly impaired gas exchange that may result in hypoxemia, pulmonary hypertension and even death. Unfortunately, little is understood about how these diseases arise, including the role of specific defects in immune tolerance. Another key question is whether autoimmune responses targeting the lung parenchyma are critical to ILD pathogenesis, including that of isolated, idiopathic forms. We show that a specific defect in central tolerance brought about by mutations in the autoimmune regulator gene (*Aire*) leads to an autoreactive T cell response to a lung antigen named vomeromodulin and the development of ILD. We found that a human patient and mice with defects in Aire develop lung pathology that is strikingly similar, demonstrating that the *AIRE*-deficient model of autoimmunity is a suitable translational system in which to unravel fundamental mechanisms of ILD pathogenesis.

Introduction

The lung is often damaged in systemic autoimmune diseases such as rheumatoid arthritis and scleroderma. These pulmonary complications of autoimmune syndromes can herald a worse prognosis for affected patients (1). Furthermore, patients with progressive lung damage can develop end-stage pulmonary fibrosis and require aggressive measures like lung transplantation.

One of the most common pulmonary manifestations of systemic autoimmune syndromes is interstitial lung disease (ILD) (2). This term comprises a heterogeneous group of disorders in which fibrosis and inflammation occur within alveolar walls or in the loose tissue surrounding peribronchovascular sheaths, interlobular septa and the visceral pleura. Little is

List of supplementary materials Materials and methods Figs. S1 to S6 References

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understood about how these lung diseases develop, including whether they arise from an autoreactive immune response to the lung parenchyma. Another unresolved issue is whether ILD that occurs in the absence of an autoimmune syndrome and instead arises as an isolated pulmonary process (i.e. idiopathic ILD) also results from defects in immunological tolerance (3,4). Given that idiopathic ILDs often exhibit the same histopathologic patterns that are observed in systemic autoimmune disorders, an immune-mediated injury targeting lung proteins may indeed be important (5).

Indirect evidence to support a role for autoimmune mechanisms in the development of idiopathic ILD comes from observational studies of patients that exhibit lung-infiltrating T cells and proinflammatory cytokines in bronchoalveolar lavage (BAL) fluid (6,7). More direct evidence is derived from reports of autoantibody and T cell responses to a variety of ubiquitously expressed proteins in patients with ILD (8–10). However, these putative antigens are not tissue specific and so it is not clear how they could cause a disease process limited to the lung. One group addressed this point by demonstrating the clonal expansion of T cells from idiopathic ILD patients in response to autologous lung tissue, although the exact stimulating proteins remain unknown (11). Thus, none of the antigen-identification studies so far have conclusively demonstrated that the proteins identified are central to disease pathogenesis.

We have taken a different approach to investigating lung autoimmunity by examining the *AIRE*-deficient model of autoimmunity. Human subjects with defects in *AIRE* develop Autoimmune Polyglandular Syndrome Type 1 (APS1), a multi-organ autoimmune disease that involves the lung in some subjects (12). Aire is a transcriptional regulator expressed primarily within the thymus in thymic medullary epithelial cells (mTECs). Aire promotes immune tolerance by driving the ectopic expression of a wide array of organ specific self-antigens in mTECs (13,14). In the absence of appropriate Aire expression, these self-antigens are not displayed in the thymus, leading to a defect in thymic deletion of autoreactive T cells (15,16). Recent work in mice has confirmed a link between thymic self-antigens and T cell responses. The multi-organ nature of disease in *AIRE*-deficient animals appears to be a result of the spectrum of self-antigens whose thymic expression relies on Aire (17,18). *Aire*-deficient mice develop lung autoimmunity that is strikingly similar in pattern to the disease reported in APS1 patients, but the specificity of this response is unknown (12,15).

We sought to determine whether lung disease in APS1 subjects can be linked to an autoimmune T cell response by detailed study of a patient with ILD and *Aire*-deficient mice. Here we demonstrate that pulmonary disease in *Aire*-deficient mice is an interstitial lung disease with features similar to patterns observed in human autoimmune disorders, including in an APS1 subject. We identify an Aire-regulated lung autoantigen that is a target of this lung-specific immune response in mice and present an APS1 patient with ILD that has autoreactivity to a similar human lung protein. Taken together, these results demonstrate that loss of tolerance to a lung autoantigen can result in autoimmune-mediated lung injury.

Results

Interstitial lung disease in *Aire*$^{o/o}$ mice and an APS1 patient

In order to determine the pattern of lung disease in *Aire*$^{o/o}$ mice, we sacrificed BALB/c, NOD and C57BL/6 (B6) *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice at various ages. The lungs were analyzed for histology by hematoxylin and eosin (H&E) staining. At early ages, the histologic pattern of disease was identical in mice in all strains. The infiltrates were comprised of mononuclear cells in a peribronchovascular distribution. Older BALB/c and NOD mice developed progressive and often severe disease. The mononuclear infiltrates extended into the lung

parenchyma and resulted in a temporally homogeneous, mild to moderate cellular interstitial pneumonia that often reached the pleural surface (Figs. 1A and S1B). In a prior study, we reported that we could induce lung disease by the adoptive transfer of *Aire*$^{o/o}$ splenocytes into immunodeficient SCID mice suggesting that the pulmonary infiltrates are autoimmune in nature (19).

APS1 patients can develop an autoimmune lung disease that is pathologically similar to the infiltrates in the mice (12). To directly the compare the histologic patterns of *Aire*$^{o/o}$ mice and APS1 subjects, we obtained a lung biopsy specimen from a patient with APS1 and a history of pulmonary disease (Fig. S2). The biopsy demonstrated mononuclear infiltrates surrounding small and medium sized airways similar to infiltrates in *Aire*$^{o/o}$ mice (Fig. 1A). The H&E stains also revealed a cellular interstitial pneumonia that mirrored findings in older NOD and BALB/c *Aire*$^{o/o}$ mice (Figs. 1A and S1B). Stains for acid fast bacilli, bacteria and fungal elements were negative.

These results demonstrate that *Aire*$^{o/o}$ mice exhibit a bronchiolitis that in older NOD and BALB/c *Aire*$^{o/o}$ animals progresses to a cellular interstitial pneumonia. This histopathology is similar to that in the biopsy from the APS1 subject with pulmonary disease.

Next we sought to identify the lung infiltrating cells that cause disease. Lungs from *Aire*$^{o/o}$ mice were prepared as frozen sections and immunohistochemistry staining was performed. Within the peribronchovascular infiltrates, the cells stained primarily for CD4, although B cells (indicated by B220 staining) were also present in significant numbers (Fig. 1B). Several mice with severe disease developed organized lymphoid structures resembling bronchus-associated lymphoid tissue (BALT) (Fig. S1A). This finding is consistent with reports identifying BALT in settings of chronic inflammation, including autoimmune disease (20). The majority of cells residing within the pulmonary interstitium were $CD4^+$ cells, while $CD8^+$ cells were present at lower numbers (Fig. 1B). Immunohistochemistry of tissue sections from the APS1 patient revealed an early germinal center with a significant number of $CD20^+$ B cells. $CD4^+$ cells appeared slightly more abundant than the $CD8^+$ cells (Fig. 1C).

Further characterization of the mouse cells by intracellular cytokine staining was performed to determine the effector mechanisms important for inducing lung disease. Lymphocytes from lungs of BALB/c and NOD *Aire*$^{o/o}$ mice were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and analyzed by flow cytometry. The cell counts confirmed that the majority of cells were $CD4^+$ T cells with B cells present in significant numbers (Fig. S3). The predominant effector cytokine produced by *Aire*$^{o/o}$ lung $CD4^+$ T cells was interferon γ (IFNγ), followed by roughly equivalent levels of IL-17A and IL-10, and low amounts of IL-4 secreting cells (Fig. 1D). These results show that lung infiltrating cells in *Aire*$^{o/o}$ mice are primarily T_H1 polarized $CD4^+$ T cells, although T_H17 and T_H2 cells are also present.

Given the predominance of lymphocytes within the lung parenchyma, we next wanted to study whether *Aire*$^{o/o}$ mice exhibited a lung-specific immune response. Serum from *Aire*$^{o/o}$ mice with lung disease was used to stain frozen lung sections from immunodeficient SCID mice to determine whether there was autoantibody reactivity to a lung protein. Reactivity was seen on the surface of the bronchiolar epithelium (Fig. 1E); less abundant staining occurred within cells located in the alveoli (Fig. 2G).

Thus, *Aire*$^{o/o}$ mice develop ILD with a histopathologic pattern similar to that seen in an APS1 patient. The lung infiltrates in *Aire*$^{o/o}$ mice are comprised of T_H1 polarized $CD4^+$ T cells, with some cells skewed toward a T_H17 phenotype. *Aire*$^{o/o}$ mice harbor autoantibodies

that target lung proteins located in the bronchiolar epithelium and alveolar cells, indicating a lung-specific immune response.

Vomeromodulin as a major lung autoantigen in *Aire$^{o/o}$* mice

We sought to identify the lung proteins targeted by autoantibodies in the serum of *Aire$^{o/o}$* mice. We performed immunoblots using lung lysate prepared from immunodeficient SCID mice that was probed with serum from B6, BALB/c and NOD *Aire$^{o/o}$* mice. All strains of *Aire$^{o/o}$* mice had serum autoantibodies to an 80 kD lung protein, whereas serum from *Aire$^{+/+}$* mice did not (Fig. 2A). Immunofluorescence staining using *Aire$^{o/o}$* serum (Fig. 1E) revealed that this target antigen was located on the surface of the bronchiolar epithelium, so we hypothesized that the protein was secreted into the airway and thus, present in BAL fluid from the lung. To test this, we performed immunoblots of BAL fluid that was probed with serum from *Aire$^{o/o}$* mice and they revealed that the 80 kD antigen was indeed present in the fluid (Fig. 2B).

To determine whether other antigens were targeted during the course of disease, we tested sera from NOD *Aire$^{o/o}$* mice that were serially bled over 4 to 10 weeks, and BALB/c *Aire$^{o/o}$* mice aged 5 to 15 weeks. The 80 kD band was the predominant target, with rare evidence of other immunoreactivity, even in older animals (Fig. 2C). Taken together, these results demonstrate that the 80 kD lung protein is likely a primary lung antigen in *Aire$^{o/o}$* mice in all strains.

Our next goal was to identify the 80 kD lung protein targeted in *Aire$^{o/o}$* mice. In a prior study, we isolated a critical eye antigen in *Aire$^{o/o}$* mice by immunoaffinity purification using *Aire$^{o/o}$* autoantibodies (17). Using similar methods, we used sera with immunoreactivity to the 80 kD protein to immunopurify the target antigen from BAL fluid. We concentrated the immunopurified protein, resolved it on a two-dimensional gel and stained it with coomassie. Four spots were detected, three of which migrated at 80 kD, and an additional spot at 60 kD (Fig. 2D). All four spots were analyzed by mass spectrometry. Peptide mapping from two of the three spots isolated at 80 kD spanned the entire amino acid sequence and were provisionally identified as vomeromodulin (UniProt accession Q80XI7–1) with a high degree of confidence (Fig. 2E). The discrepancy between the predicted molecular weight of vomeromodulin (VM) at 62 kD and the protein on immunoblots at 80 kD appears to be due to glycosylation, which causes VM to migrate at a higher molecular weight (Fig. S4). The 60 kD spot was identified as albumin, which is abundant in BAL fluid and probably non-specifically bound to our immunoprecipitation column.

To confirm that the provisional identification of vomeromodulin corresponded to the 80 kD protein on immunoblots, we generated recombinant VM protein coupled to a maltose binding protein (MBP) tag. A competition assay performed using recombinant VM (VM-MBP) revealed that the 80 kD serum reactivity on immunoblots was abolished with the addition of 0.25 μg of recombinant protein. As a control, we added up to 16 μg of the MBP tag to the same serum and failed to abrogate the 80 kD reactivity (Fig. 2F). Further verification that VM was our target antigen came from co-staining experiments with *Aire$^{o/o}$* serum and a rat VM anti-serum. (The homology between rat and mouse VM amino acid sequences is greater than 80%). Merged images of staining with the two antibodies show they co-localized to the apical surface of the bronchiolar epithelium and to rare cells within lung alveoli (Fig. 2G).

In summary, we identified vomeromodulin as a major lung autoantigen in *Aire$^{o/o}$* mice, confirming the presence of a lung autoimmune response in our model.

Aire$^{o/o}$ mice have T cells specific for the Aire-regulated protein vomeromodulin

Vomeromodulin is enriched in the respiratory epithelium of the rat (21). To clarify the tissue distribution of VM in the mouse, we used cDNA libraries of several tissues and performed RT-PCR for full length VM cDNA. We could only amplify VM cDNA from lung cDNA (Fig. 3A). We excised the PCR product, sequenced the DNA and confirmed that we had isolated the full coding region of VM. To determine the tissue content of the VM protein, we performed immunoblotting on protein lysates of different organs (Fig 3B). We probed the tissues with a serum sample that had proven reactivity to vomeromodulin as demonstrated through competition immunoblots (Fig. 2F). In the multi-organ immunoblot, we detected 80 kD reactivity only in lung lysate. Taken together, these data confirm that mouse VM has a restricted expression pattern that is mainly limited to the respiratory epithelium.

Given that *Aire*$^{o/o}$ mice generate a VM-specific immune response and exhibit CD4^{+} T cells within lung infiltrates, we hypothesized that VM was expressed in the thymus in an Aire-dependent manner. In this scenario, the absence of VM thymic expression in *Aire*$^{o/o}$ mice might allow the escape of VM-specific T cells that induce lung disease. To test this, we ran real-time PCR for VM on cDNA from purified thymic stroma of *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice. Our analysis revealed that VM thymic expression was indeed Aire dependent, as was insulin, a known Aire-regulated antigen (Fig. 3C). As an additional control, we tested GAD67 and confirmed that this Aire-independent tissue specific protein was expressed equally in thymi from *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice (Fig. 3C) (13). We then determined whether there was an increase in the number of IFNγ-producing T cells with specificity for VM in *Aire*$^{o/o}$ mice. We assayed CD4^{+} T cells from *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice for VM specificity using an ELISPOT analysis (Fig 3D). We found that *Aire*$^{o/o}$ mice had a statistically significant increase in the number of IFNγ-producing, VM-specific T cells. These data demonstrate that an *Aire*-mediated defect in the development of tolerance in the thymus likely leads to the release of VM-specific T cells capable of inducing lung-specific tissue damage.

Breaking tolerance to VM through adjuvant immunization

Negative selection of self-reactive T cells during development purges the immune repertoire of autoreactive cells, but the process may not be complete. T cells specific for self-antigens can be detected in healthy humans but do not cause overt autoimmunity as peripheral tolerance mechanisms keep these cells from becoming activated and causing disease (22). To determine if VM is a major lung autoantigen that by itself can induce pulmonary disease, we devised an immunization protocol modeled after the experimental multiple sclerosis model, Experimental Autoimmune Encephalomyelitis (EAE) (23). Using complete Freund's adjuvant (CFA), we immunized wild-type BALB/c mice with VM-MBP, followed by two additional rounds of immunization using VM-MBP in incomplete Freund's adjuvant (IFA). The mice were sacrificed and their organs analyzed for histology with H&E staining. The VM-MBP immunized mice developed lung disease similar to the spontaneous disease in *Aire*$^{o/o}$ mice and this disease was limited to the lung (Fig. 4A). A cohort of BALB/c wild-type mice immunized with the MBP protein tag with the same protocol did not develop lung disease (Fig. 4B). We tested the sera from immunized mice for VM antibodies and showed VM-specific immune reactivity in all of the VM immunized mice but not in the MBP-immunized controls (Fig. 4C). Thus, we demonstrate that inducing a break in tolerance with adjuvant immunization can activate VM-specific cells to cause a lung-restricted disease similar to the spontaneous pulmonary infiltrates observed in *Aire*$^{o/o}$ mice.

Lung specific disease from the adoptive transfer of activated VM-specific cells

One hallmark of autoimmune disease is that the adoptive transfer of activated antigen-specific cells to another host transfers the disease. We therefore devised a protocol for the

adoptive transfer of VM-specific immune cells harvested from immunized wild-type mice. To facilitate *in vitro* activation of the cells, we generated VM peptides predicted by computer modeling to bind to BALB/c MHC Class II, I-A^d (24). Using ELISPOT analysis, we screened the peptides to determine whether *Aire*$^{o/o}$ mice had T cells specific for them. We found that *Aire*$^{o/o}$ mice had IFNγ-producing T cells specific for the 15 amino acid peptide NLEGMLADVLNTVES (Fig. S5). This VM peptide, which we named VM-111, was used to immunize wild-type BALB/c mice and *in vitro* activate spleen and lymph node cells from these mice in conditions favoring the growth of $CD4^+$ T cells. A peptide derived from chicken ovalbumin (Ova) that is also known to bind I-A^d was used as our negative control for the disease transfer (Fig. 5A). A portion of cells from immunized mice were analyzed by [H^3] thymidine incorporation to confirm that cells proliferated in response to our antigen (Fig. 5B). We adoptively transferred 20×10^6 activated cells into each of our immunodeficient SCID mice and sacrificed them 4 to 6 weeks later. The resulting lung disease was less severe than the spontaneous disease of *Aire*$^{o/o}$ mice, but the infiltrates were limited to the lung (Fig. 5CD) and had a similar appearance. Our results show that the adoptive transfer of activated VM-specific cells can transfer lung disease and that the likely pathogenic effectors are $CD4^+$ T cells.

Autoreactivity to LPLUNC1, a VM-like protein, in a patient with APS1

Given our data that supports an autoreactive lung response in *Aire*$^{o/o}$ mice, we tested for a similar response in an APS1 patient with lung disease. Although we cannot rule out contributing factors such as aspiration for the patient's pulmonary disease, we sought to determine whether the patient exhibited an autoimmune response to lung tissue by staining frozen sections of normal human lung with the patient's serum (Fig. 6A). The pattern of immunoreactivity was nearly identical to the pattern of staining seen using sera from *Aire*$^{o/o}$ mice (Figs. 1E, 6A). We tested the patient's serum for autoantibodies to KCNRG, a bronchial epithelial antigen recently identified in APS1 (12), but failed to demonstrate any autoreactivity (Fig. S6).

A direct human homolog of mouse VM does not appear to exist. On the basis of surveys of the University of California Santa Cruz and Ensembl genome databases, the human gene transcript orthologous to VM is C20orf115 and is likely an unprocessed pseudogene. The organization of the human genomic region containing C20orf115 is similar to that of the mouse, and it contains the PLUNC family of proteins, including LPLUNC1 (C20orf114), the adjacent transcript upstream from the human VM pseudogene (Fig. 6C). An analysis of the domain structure of VM using the NCBI Conserved Domain Database revealed that the VM protein shares the BPI (Bactericidal/Permeability Increasing protein) superfamily domain with the PLUNC (Palate, Lung, and Nasal epithelium Carcinoma associated protein) family of proteins located in the same region on mouse chromosome 2 (Figs. 6D–E) (25). The PLUNC proteins have not previously been implicated in autoimmunity, but, because of their similar domain structure and expression pattern to VM, we tested our APS1 patient serum against LPLUNC1, the family member with the highest levels of gene expression in the lung, in particular the bronchiolar epithelium (26,27). An autoantibody assay revealed that our APS1 patient with lung disease had immunoreactivity to LPLUNC1 that was not seen in our healthy controls or other samples from APS1 patients without lung disease (Fig. 6F). Immunofluorescent staining of normal human lung tissue with a human LPLUNC1 antibody showed that the protein is located on the bronchiolar epithelium in a pattern similar to the LPLUNC1 distribution in the APS1 patient (Fig. 6G–H). Our data shows that the lung disease in an APS1 patient closely recapitulates the lung disease in *Aire*$^{o/o}$ mice, suggesting that LPLUNC1 may be an important human lung autoantigen.

Discussion

Here we have shown that a loss of immune tolerance to a lung self antigen results in spontaneous interstitial lung disease. We used a relatively unbiased approach to identify the lung protein vomeromodulin as a key autoantigen in spontaneous ILD that occurs in several strains of *Aire*$^{o/o}$ mice. We demonstrated that immune responses directed against VM are sufficient to induce lung autoimmunity and found that VM is expressed within the thymus in an Aire-dependent manner. This places VM among similar organ specific autoantigens previously identified in the *Aire*-deficient model and illustrates the importance of central tolerance in the prevention of ILD. Our results show how an immune response against a lung antigen can cause ILD both in the setting of multi-organ autoimmunity and as an isolated organ-specific disease. We extended these findings to an APS1 patient with lung disease that histologically mirrors ILD in the mouse and demonstrated autoreactivity to a human antigen that is similar to VM, providing evidence for a potential diagnostic biomarker and therapeutic target in this subset of patients.

To date it has been difficult to conclusively show that autoimmune mechanisms play an important role in ILD pathogenesis. Several studies report the presence of activated lymphocytes in the lung or lavage fluid of ILD patients, but the underlying cause for their presence is unknown (7,28). Given the lung's extensive vascular network and exposed mucosal surface, there are numerous factors that may drive immune cells to the lung in ILD. Hypotheses for this range from infectious causes, vascular injury or a hypersensitivity reaction to inhaled antigens (5). We have now demonstrated that an autoimmune response to a lung self-antigen can provide a sufficient trigger for interstitial lung disease. Characterization of the cells infiltrating the lung showed that many are T_H1 polarized $CD4^+$ T cells (Fig. 1D), consistent with a human study that identified a similar phenotype in patients with idiopathic ILD (7).

The lymphocyte accumulation in lung resulted in a bronchiolitis and cellular interstitial pneumonia in *Aire*$^{o/o}$ mice that was similar to the lung disease in the APS1 patient and in APS1 patients described in the literature (12). Importantly, these histologic patterns are also frequently seen in other human autoimmune syndromes (3,5). With our animal model we can define the natural history of ILD, a task that has been difficult to accomplish in patients. For instance, the airway-centered infiltrates appear to precede interstitial pneumonia in *Aire*$^{o/o}$ mice, suggesting that a similar temporal pattern may occur in ILD patients since these features are sometimes seen simultaneously in human disease (4).

Aire-mediated control of peripheral tissue antigen expression in the thymus is a fundamental mechanism by which immune tolerance to self-antigens is established. We showed that the thymic expression of our lung autoantigen is controlled by Aire. In *Aire*$^{o/o}$ mice, the VM transcript in the thymus was nearly absent (Fig. 3C), which likely facilitates the escape of VM-specific T cells from negative selection and their subsequent release into the periphery (Fig. 3D). In support of a T cell response to VM in lung disease, we showed that the adoptive transfer of activated VM-specific cells was capable of inducing lung infiltrates. Previous work confirms that Aire-mediated control of tissue-specific self-antigens is critical for the prevention of organ specific autoimmune diseases. We reported in a prior study that loss of the Aire-regulated eye autoantigen, interphotoreceptor retinoid-binding protein (IRBP), in the thymus is sufficient to induce a T cell-mediated autoimmune uveitis (17). Another study identified the Aire-dependent protein seminal vesicle secretory protein 2 (SVS2) as a primary target of a T cell-mediated autoimmune prostatitis (18). Despite the different expression patterns of these tissue-specific antigens, a notable feature linking VM, IRBP and SVS2 is that they are all secreted into the extracellular space. We speculate that this commonality is due to their increased accessibility as autoantigens in the periphery,

although Aire does not appear to preferentially regulate the transcription of secreted proteins (29,30). It should also be noted that the immunogenicity of secreted antigens could potentially be offset by dendritic cells that pick up the protein and traffic to the thymus (31). Nevertheless, the expression and presentation of tissue antigens to developing thymocytes is an important component of the immune tolerance mechanism mediated by Aire, and this pathway is likely important in the control of autoimmune diseases other than APS1. For example, in Type 1 Diabetes and myasthenia gravis the thymic abundance of the target autoantigens (insulin and the α-subunit of the muscle acetylcholine receptor, respectively) correlates with susceptibility to disease (32,33). This same principle may govern the abundance of thymic lung antigens and determine the propensity of APS1 patients and other patients with autoimmune disease to develop ILD.

There are no reports implicating vomeromodulin in autoimmunity and much remains unknown about the protein and its function. Vomeromodulin was discovered in the lateral nasal glands of the rat, where it is secreted into the mucus overlying the respiratory epithelium of the nasal passages, including the vomeronasal organ, and primarily localized to the olfactory epithelium (21). Given this distribution the authors hypothesized the protein might be involved in pheromone transport to the vomeronasal organ. Similar to VM, the PLUNC proteins are BPI homologs that are secreted into the mucus covering the respiratory epithelium of larger airways. They are thought to have antimicrobial properties and be important for innate immune responses (34,35). Recombinant BPI peptides may exhibit anti-fungal properties (34), and one might speculate that autoimmune responses against PLUNCs may induce a specific immunodeficiency for fungal infections. Indeed, APS1 patients frequently develop candidiasis.

Our work follows a paper that provides evidence for lung-specific autoimmunity in a subset of APS1 patients with antibodies to KCNRG, an antigen that is also expressed in the bronchial epithelium. Nearly all of the patients with autoantibodies to KCNRG had pulmonary disease, defined broadly as respiratory symptoms. Three children underwent lung biopsies, which demonstrated a lymphocytic infiltrate surrounding small and large airways that was similar to the early disease we saw in *Aire*$^{o/o}$ mice (12). The function of the KCNRG protein is not currently understood; structurally it has little resemblance to VM or LPLUNC1. By immunofluorescence the distribution of KCNRG in the lung mirrors that of both LPLUNC1 and VM. We did not detect autoreactivity to a protein that migrated at the weight of mouse KCNRG on immunoblotting with *Aire*$^{o/o}$ serum. We also did not isolate KCNRG from our immunoaffinity purification, and KCNRG does not appear to be a major autoantigen in the mouse system. Nevertheless, it is clear that bronchiolar epithelium is a primary target for autoimmune reactions, and it will be interesting to determine whether APS1 patients with KCNRG reactivity also have autoantibodies to the LPLUNC1 antigen.

Finally, an important outcome of our work is the development of an inducible autoimmune lung disease model through immunization to the self-antigen VM. This system could be utilized similarly to the EAE model of multiple sclerosis. The EAE system has been invaluable in demonstrating the many effector pathways in autoimmune central nervous system disease such as the recently highlighted IL-17 secreting T cells (36). Our ability to cause lung disease through adjuvant immunization suggests that Aire-mediated tolerance to the VM protein is not complete. The low numbers of VM-specific T cells we identified in wild-type mice (Fig. 3D) indicates that other tolerance mechanisms may be preventing these cells from becoming activated and causing disease. This may serve as a clue to a process by which even subtle defects in central tolerance can lead to the development of ILD, possibly as a cumulative result of multiple factors including, for instance, a failure of peripheral tolerance mechanisms or a lung insult caused by inhalational injury. Further investigation will be needed to explore the potential role of autoimmune responses to lung antigens in

interstitial lung diseases. Taken together, our results here provide a framework by which these diseases may arise and the likely role of central tolerance in preventing them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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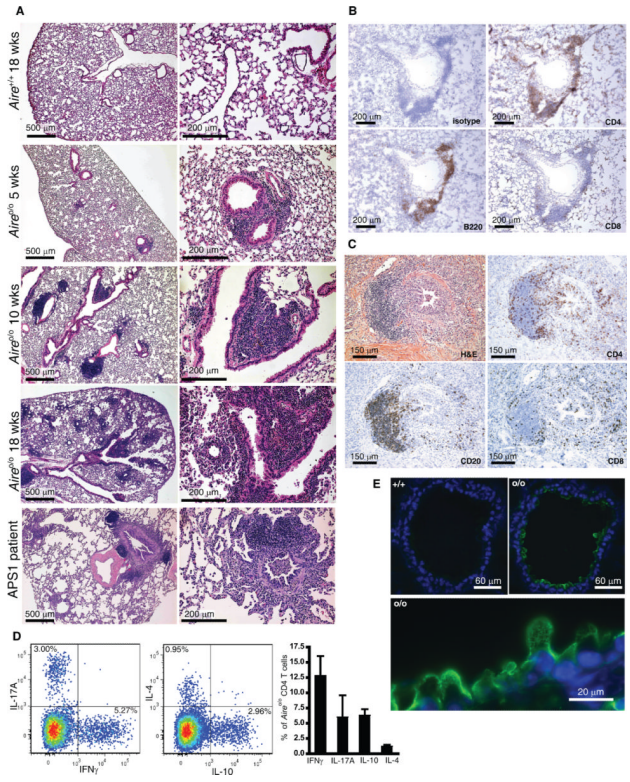

Fig. 1. Pulmonary disease in *Aire*$^{o/o}$ mice and an APS1 patient

(A) Representative H&E of lungs from BALB/c *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice. (Lower panels) H&E of lung biopsy from the APS1 patient. (B) Immunostaining of lungs from NOD *Aire*$^{o/o}$ mouse at 8 weeks for CD4, CD8, B cells and isotype control antibody. (C) Lung biopsy from an APS1 patient shows chronic bronchiolitis and prominent lymphoid aggregates. Immunostaining shows a nodular aggregate of B-cells (CD20^{+}) consistent with early germinal center formation and scattered surrounding CD4^{+} and CD8^{+} T cells. (D) Representative plot of CD4^{+} lung lymphocytes from a BALB/c *Aire*$^{o/o}$ mouse at 14 weeks, showing IL-17A, IFNγ, IL-4 and IL-10 containing cells. (Right panel) Percentages of total CD4^{+} lung lymphocytes producing cytokines averaged from 5 BALB/c *Aire*$^{o/o}$ mice aged 12–16 weeks. Data are mean ± SEM. (E) Indirect immunofluorescence stain with serum from a NOD *Aire*$^{o/o}$ mouse with pulmonary disease on frozen lung section from immunodeficient SCID mouse. (Lower panel) Higher magnification image of lung section shown in upper panel, right. Green, serum staining; blue, staining with nuclear marker 4',6'-diamidino-2-phenylindole (DAPI).

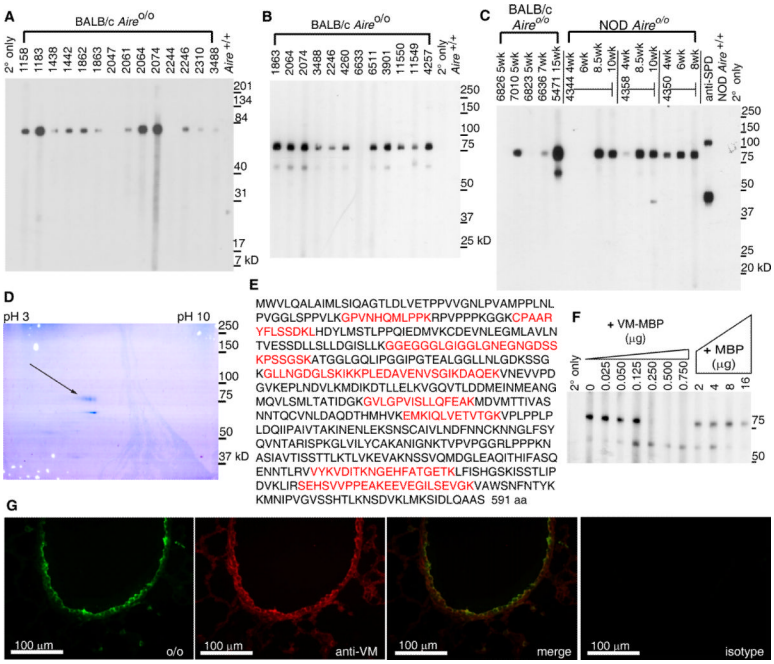

Fig. 2. Vomeromodulin, the predominant antigen targeted in lungs of *Aire*$^{o/o}$ mice

(A) Immunoblot of whole lung lysate probed with sera from BALB/c *Aire*$^{o/o}$ mice aged 8–20 weeks (individual animals numbered) revealed an 80 kD antigen target. (B) Immunoblot of BAL fluid probed with sera from BALB/c *Aire*$^{o/o}$ mice also revealed the 80 kD antigen. (C) Immunoblot of BAL fluid probed with NOD mice bled serially and BALB/c mice sacrificed at various ages. (D) Sera from *Aire*$^{o/o}$ mice were used to immunoprecipitate the antigen from BAL fluid, which was then run on a coomassiestained 2D gel. Three spots at 80 kD migrated near an isoelectric point ~5.5, (arrow). All spots were analyzed by mass spectrometry (E) The sequence of the 80 kD spot, indicating that it is vomeromodulin. Identified peptides (in red) mapped onto the VM amino acid sequence revealed coverage of nearly the entire protein. (F) To confirm autoantibody reactivity to VM, a competition blot showed 80 kD reactivity was abolished after addition of recombinant VM-MBP. The MBP tag alone failed to abolish reactivity. (G) *Aire*$^{o/o}$ serum and anti-VM sera co-localized in indirect immunofluorescence staining on lung tissue targeting the bronchiolar epithelial surface and less frequently, to cells within distal airways. SPD = surfactant protein D.

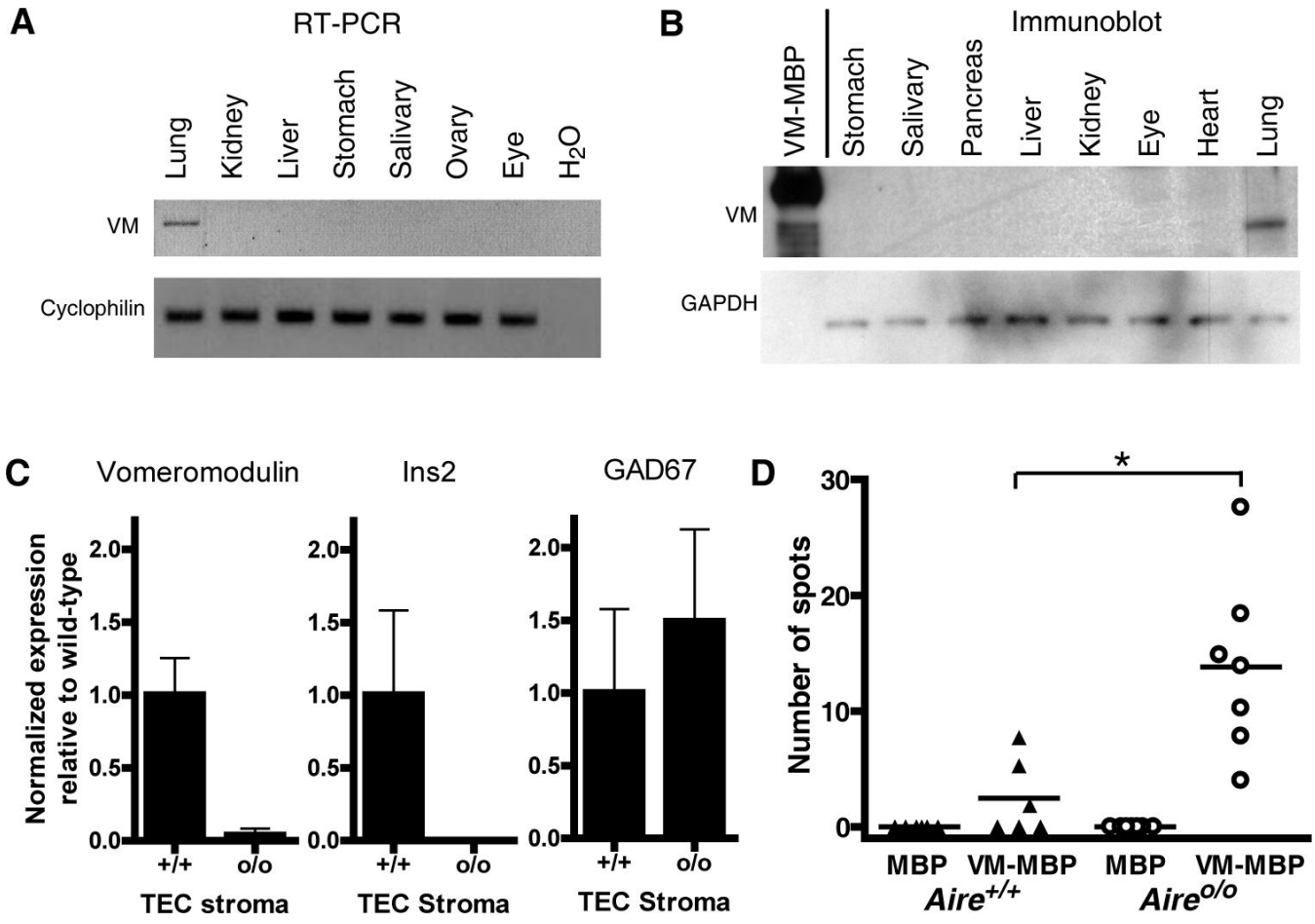

Fig. 3. T cells with specificity for vomeromodulin in *Aire*$^{o/o}$ mice

(A) RT-PCR of vomeromodulin cDNA after 35 cycles in indicated tissues reveals a band at expected size of 1.8 kb in lung only. The DNA band was excised and sequenced, confirming that full length VM cDNA was amplified. (B) Immunoblot using BALB/c *Aire*$^{o/o}$ serum to probe tissue lysates of indicated organs and recombinant VM-MBP. Reactivity to the 80 kD band is only seen in lung lysate. Reactivity to VM-MBP occurs at expected weight of 100 kD. (C) Representative results from two independent experiments in which TEC stroma from *Aire*$^{o/o}$ and *Aire*$^{+/+}$ thymi was assayed in quadruplicate for VM, insulin 2 (Ins2) and glutamic acid decarboxylase 67 (GAD67) by real-time PCR; data are normalized expression relative to wild-type ±SD. (D) ELISPOT analysis of IFNγ producing T cells in *Aire*$^{o/o}$ and *Aire*$^{+/+}$ BALB/c mice aged 10–14 weeks (*P=.005). Y axis indicates number of spots per 10,000 T cells.

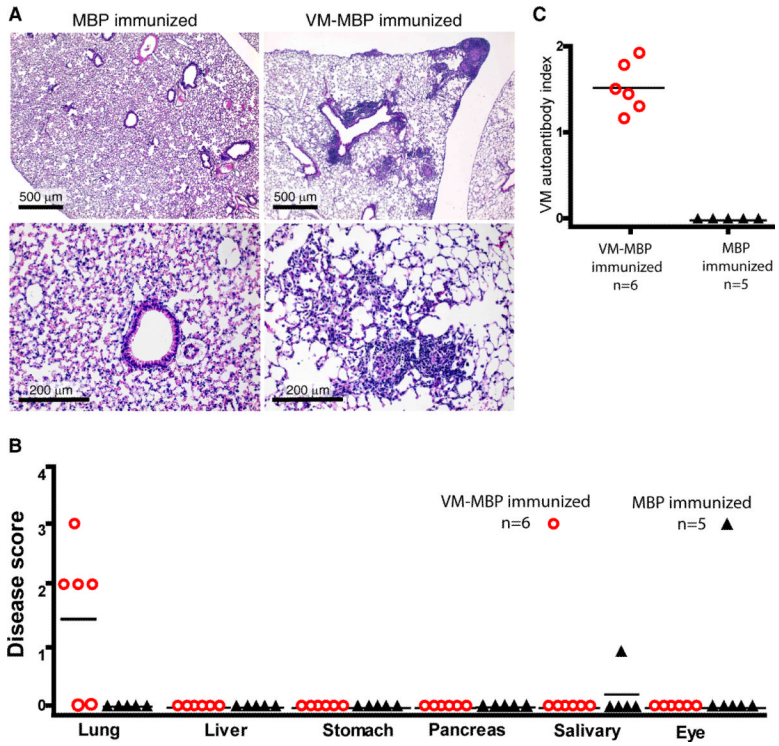

Fig. 4. Induction of lung-specific disease by breaking tolerance to vomeromodulin in wild-type mice

(A) H&E of lungs from BALB/c WT mice immunized with VM-MBP or MBP. (B) Four of six mice immunized with VM-MBP exhibited lung disease, scored as shown. Mononuclear cell infiltrates were limited to the lung, except one mouse immunized with MBP with salivary infiltrates. Line, mean disease score. (C) VM autoantibody assay showed a VM-specific immune response in mice immunized with VM-MBP but not in the MBP immunized controls.

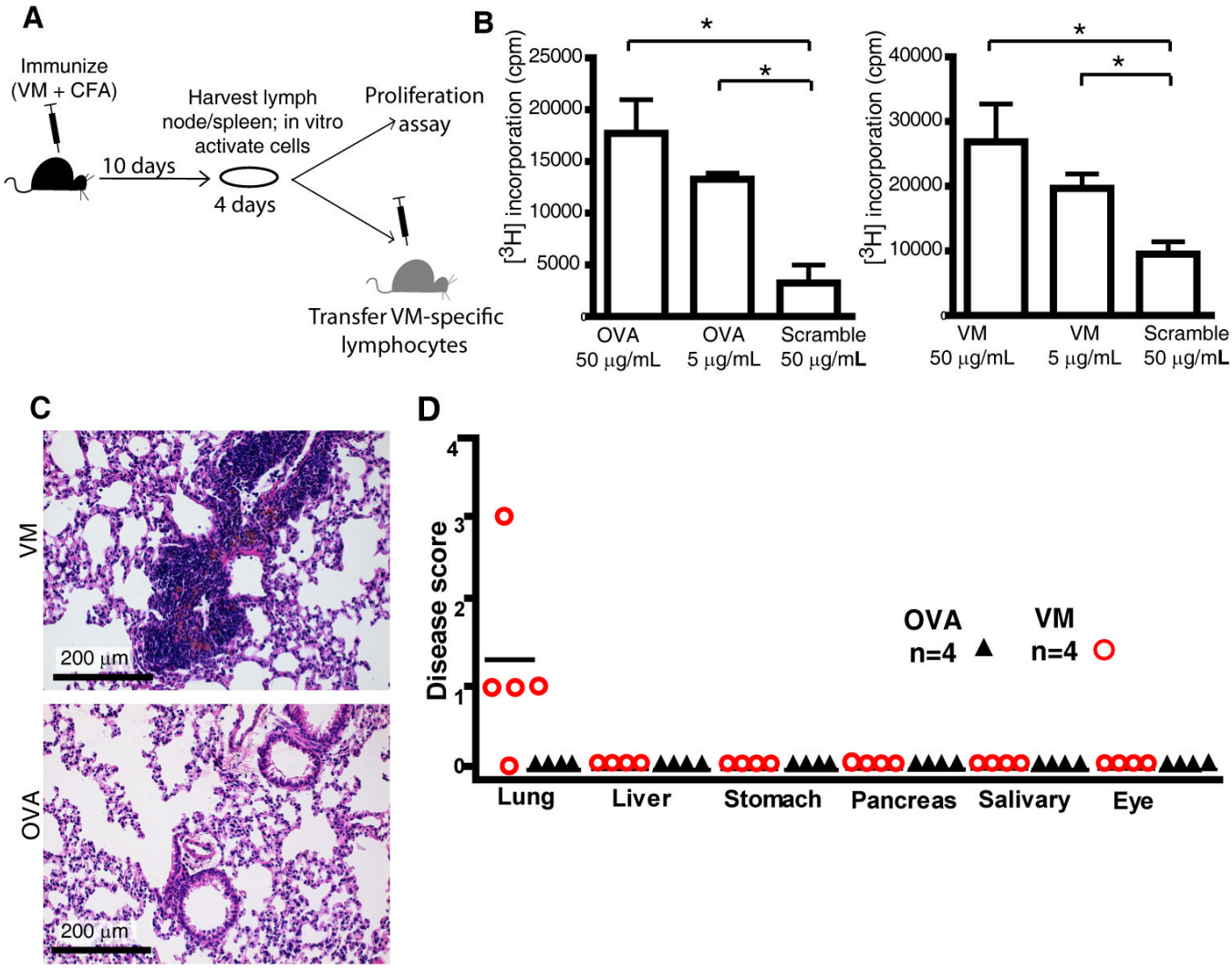

Fig. 5. Lung-specific disease after adoptive transfer of VM-specific T cells

(A) Protocol for adoptive transfer begins with immunization of BALB/c WT mice with VM or Ova peptide. Ten days later, lymph node and spleen cells are activated *in vitro* with respective peptides. Activated cells are analyzed in a proliferation assay or transferred into BALB/c SCID mice. (B) Representative [H^3] thymidine incorporation assay in cells harvested from immunized mice. Each condition performed in triplicate; data are mean ±SEM. The differences between Ova or VM peptides and scramble controls are statistically significant (*$P<0.05$ for all comparisons, two tailed t-test). (C) Mice receiving VM specific lymphocytes were sacrificed 4–6 weeks post transfer and organs analyzed for histology by H&E staining. Lung images reveal a mononuclear peribronchovascular infiltrate in the mouse receiving VM specific cells. (D) Disease scores of mice after adoptive transfer (AT) of antigen specific cells.

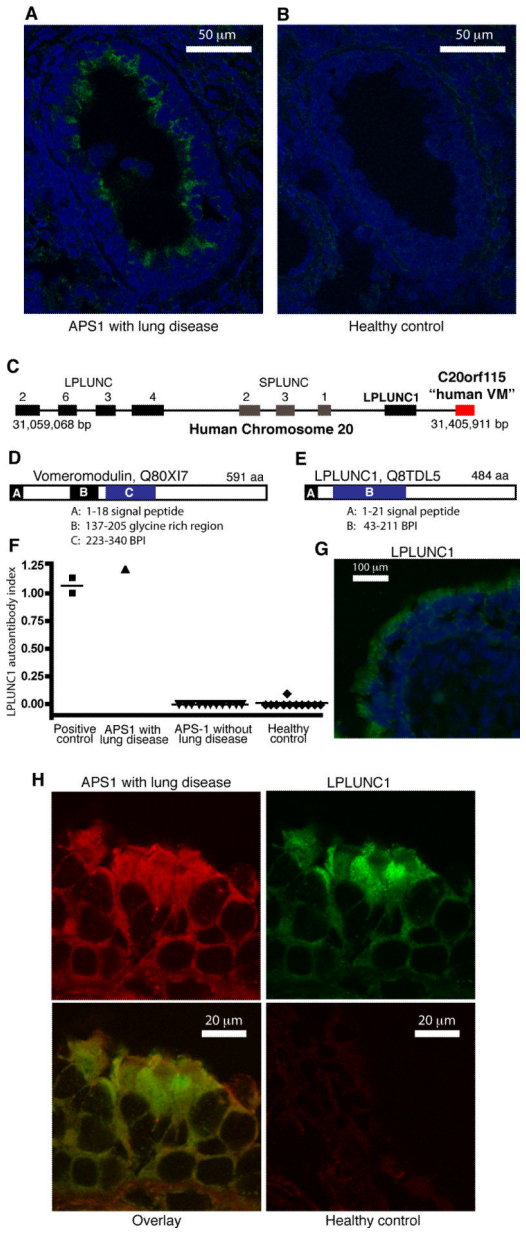

Fig. 6. Autoreactivity to a human bronchial epithelial protein, LPLUNC1, in a patient with APS1 and lung disease

(A) Immunofluorescence stain of normal frozen human lung with serum from an APS1 patient with lung disease or (B) a normal healthy patient. Green, serum staining; blue, staining with DAPI. (C) Genomic organization of the human VM pseudogene locus (C20orf115) shows the adjacent human PLUNC gene family with individual genes numbered as indicated. (D) Domain structure of murine vomeromodulin protein showing the BPI domain. (E) Domain structure of human LPLUNC1, also with a BPI domain. (F) Autoantibodies to LPLUNC1 in serum from an APS1 patient with lung disease (n=1), healthy controls (n=11) and APS1 patients without lung disease (n=11) were detected in an autoantibody assay run in triplicate using in vitro transcribed and translated, radiolabeled human LPLUNC1 protein. As a positive control, two commercial anti-human LPLUNC1 antibodies were run. Shown are representative results from 2 independent experiments. (G) Normal frozen human lung stained by immunofluorescence with antibody to human

LPLUNC1. (H) High magnification view of normal frozen human lung after immunofluorescence stain with serum from the APS1 patient with lung disease (top left, red) and the LPLUNC1 antibody (top right, green) show co-localization (bottom left) on the bronchiolar epithelium. A serial lung section stained with healthy patient serum does not demonstrate autoreactivity.