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Sci Transl Med **1**, 9ra20 (2009);
DOI: 10.1126/scitranslmed.3000284

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INTERSTITIAL LUNG DISEASE

Identification of an Autoantigen Demonstrates a Link Between Interstitial Lung Disease and a Defect in Central Tolerance

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(Published 2 December 2009; Volume 1 Issue 9 9ra20)

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 similar lung pathology, 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 such as 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 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 (that is, 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 studies 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 multiorgan 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 multiorgan 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 ILD with features similar to patterns observed in human autoimmune disorders, including in an APS1 subject. We identify an Aire-regulated lung autoantigen that

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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. Together, these results demonstrate that loss of tolerance to a lung autoantigen can result in autoimmune-mediated lung injury.

RESULTS

ILD in *Aire*$^{o/o}$ mice and an APS1 patient

To determine the pattern of lung disease in *Aire*$^{o/o}$ mice, we killed BALB/c, non-obese diabetic (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 composed 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 (Fig. 1A and fig. S1B). In a previous study, we reported that we could induce lung disease by the adoptive transfer of *Aire*$^{o/o}$ splenocytes into severe combined 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 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 (Fig. 1A and fig. 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 cel-

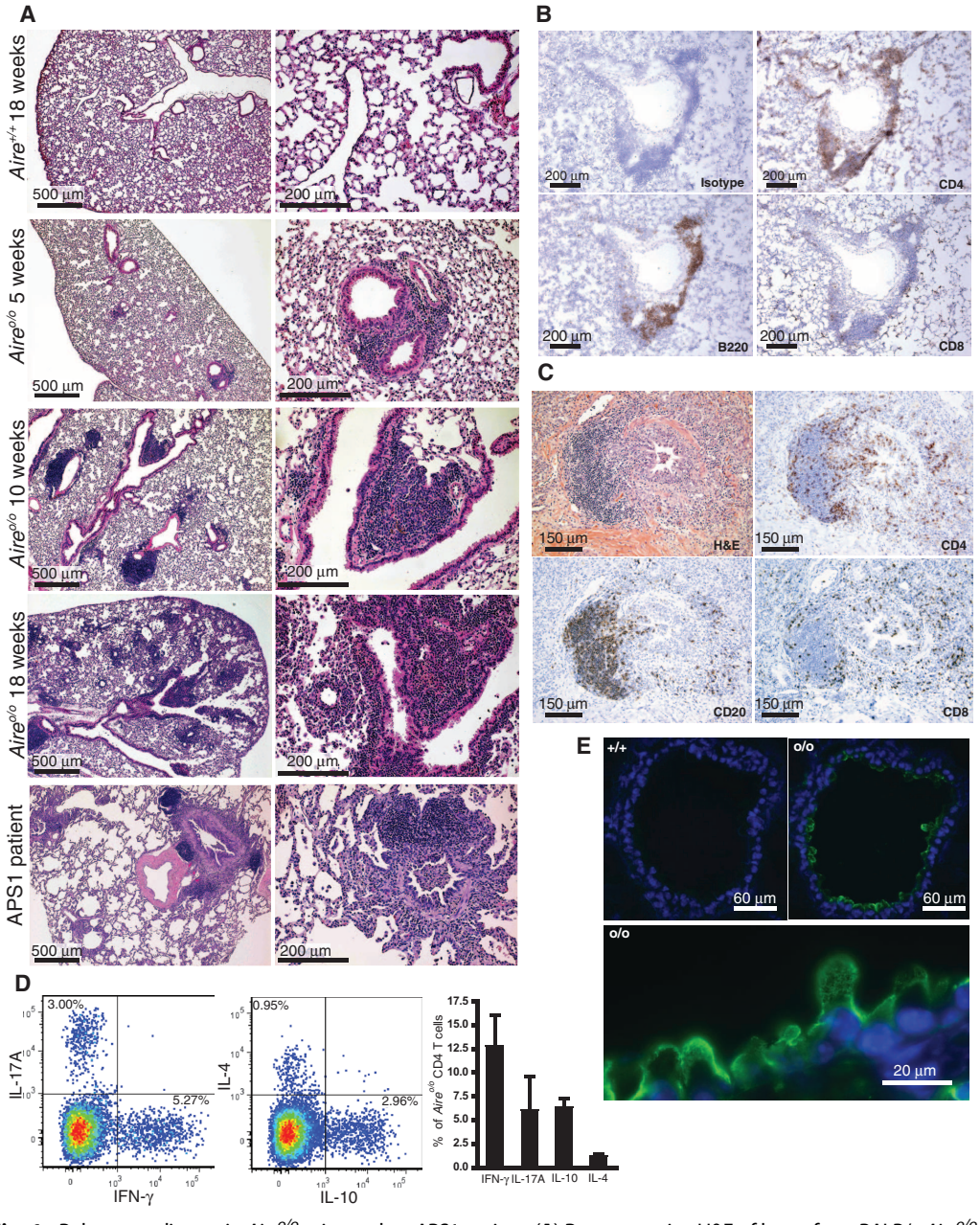


Fig. 1. Pulmonary disease in *Aire*$^{o/o}$ mice and an APS1 patient. (**A**) Representative H&E of lungs from BALB/c *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice. Lower panels, H&E of lung biopsy from the APS1 patient. (**B**) Immunostaining of lungs from NOD *Aire*$^{o/o}$ mouse at 8 weeks for CD4, CD8, 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 cells containing IL-17A, IFN-γ, IL-4, and IL-10. Right panel, percentages of total CD4$^+$ lung lymphocyte–producing cytokines averaged from five BALB/c *Aire*$^{o/o}$ mice aged 12 to 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 the right upper panel. Green, serum staining; blue, staining with nuclear marker 4′,6-diamidino-2-phenylindole (DAPI).

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lular 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 immunohistochemical 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 studies identifying BALT in settings of chronic inflammation, including autoimmune disease (*20*). The majority of cells residing within the pulmonary interstitium were CD4$^+$ cells, whereas 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 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 (IFN-γ), followed by roughly equivalent concentrations of interleukin-17A (IL-17A)– and IL-10–secreting cells and low concentrations of IL-4–secreting cells (Fig. 1D). These results show that the lung-infiltrating cells in *Aire*$^{o/o}$ mice are primarily T helper 1 (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 composed of T_H1-polarized CD4$^+$ T cells, with some cells skewed toward a T_H17 phenotype. *Aire*$^{o/o}$ mice harbor autoantibodies that target *Aire*$^{o/o}$ serum and antibody to VM sera colocalized in indirect immunofluorescence staining on lung tissue targeting the bronchiolar epithelial surface and less frequently to cells within distal airways.

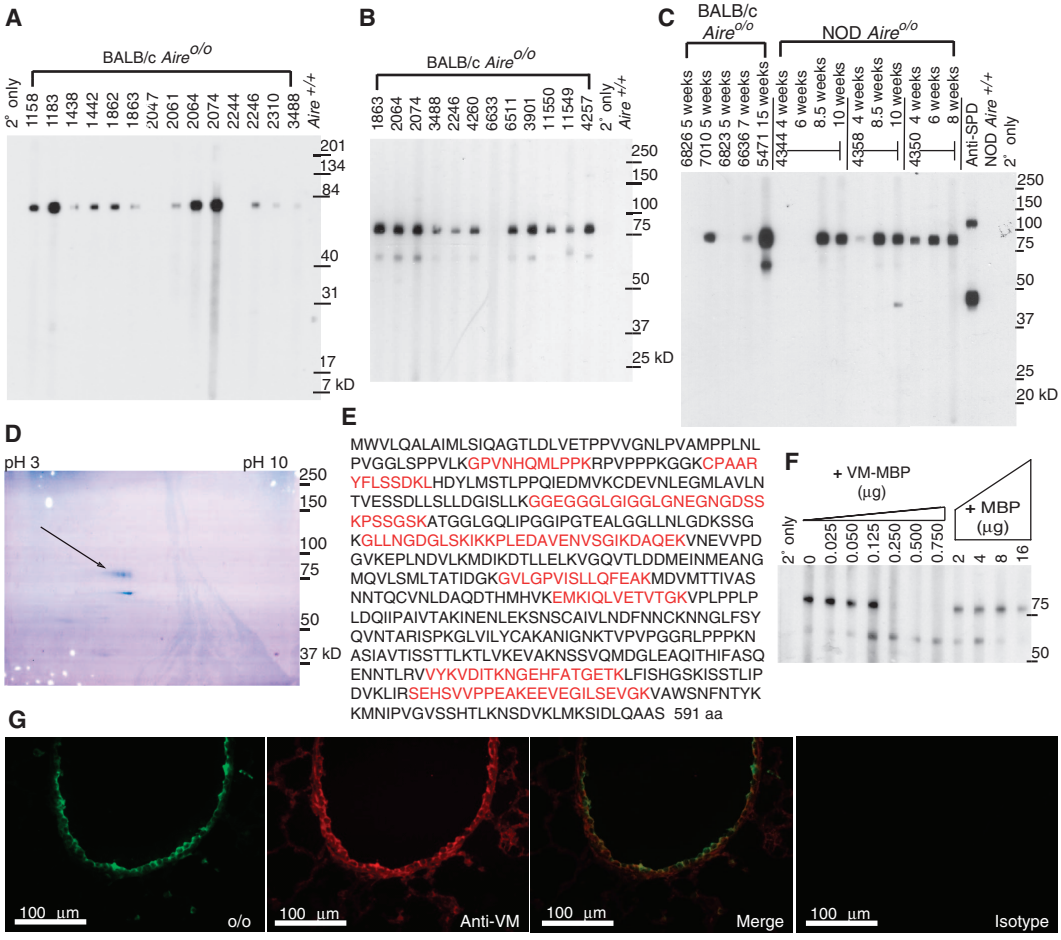


Fig. 2. VM, the predominant antigen targeted in lungs of *Aire*$^{o/o}$ mice. (**A**) Immunoblot of whole lung lysate probed with sera from BALB/c *Aire*$^{o/o}$ mice aged 8 to 20 weeks (individual animals numbered) revealed an 80-kD antigen target. (**B**) Immunoblot of BAL fluid probed with sera from 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 killed at various ages. SPD, surfactant protein D. (**D**) Sera from *Aire*$^{o/o}$ mice were used to immunoprecipitate the antigen from BAL fluid, which was then run on a Coomassie-stained two-dimensional gel. Arrow, three spots at 80 kD migrated near an isoelectric point of ~5.5. All spots were analyzed by mass spectrometry. (**E**) The sequence of the 80-kD spot, indicating that it is VM. Identified peptides (red) that mapped onto the VM amino acid (aa) sequence revealed coverage of nearly the entire protein. Abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**F**) To confirm autoantibody reactivity to VM, a competition blot showed that 80-kD reactivity was abolished after addition of recombinant VM-MBP. The MBP tag alone failed to abolish reactivity. (**G**)

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get 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. Therefore, 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). 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 previous 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 autoreactivity to the 80-kD protein to immunopurify the target antigen from BAL fluid.

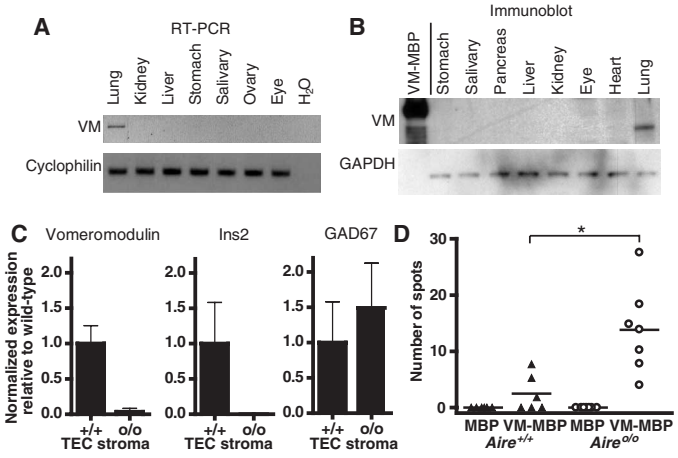


Fig. 3. T cells with specificity for VM in *Aire*$^{o/o}$ mice. (**A**) RT-PCR of VM cDNA after 35 cycles in indicated tissues reveals a band at expected size of 1.8 kb in lung only. The DNA band was excised and sequenced, confirming that full-length VM cDNA was amplified. (**B**) Immunoblot using BALB/c *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 GAD67 by real-time PCR; data are normalized expression relative to wild type ± SD. (**D**) ELISPOT analysis of IFN-γ–producing T cells in *Aire*$^{o/o}$ and *Aire*$^{+/+}$ BALB/c mice aged 10 to 14 weeks. *$P = 0.005$. *Y* axis, number of spots per 10,000 T cells.

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 these spots were provisionally identified as vomeromodulin (VM) (UniProt accession Q80XI7-1) with a high degree of confidence (Fig. 2E). The discrepancy between the predicted molecular size of 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 size (fig. S4). The 60-kD spot was identified as albumin, which is abundant in BAL fluid and probably nonspecifically bound to our immunoprecipitation column.

To confirm that the provisional identification of VM 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 with 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 costaining experiments with *Aire*$^{o/o}$ serum and a rat VM antiserum (the homology between rat and mouse VM amino acid sequences is >80%). Merged images of staining with the two antibodies show that they colocalized to the apical surface of the bronchiolar epithelium and to rare cells within the lung alveoli (Fig. 2G).

In summary, we identified VM 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 VM

VM is enriched in the respiratory epithelium of the rat (*21*). To clarify the tissue distribution of VM in the mouse, we used RNA from several tissues and performed reverse transcription polymerase chain reaction (RT-PCR) for full-length VM cDNA. We could only amplify VM cDNA from the lung (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 distribution of VM protein expression, we performed immunoblotting on lysates of different organs (Fig. 3B). We probed the tissues with a serum sample that had proven reactivity to VM as demonstrated through competition immunoblots (Fig. 2F). In the multiorgan immunoblot, we detected 80-kD reactivity only in lung lysate. 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 glutamic acid decarboxylase 67 (GAD67) and confirmed that this Aire-independent, tissue-specific protein was expressed equally in thymi

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from *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice (Fig. 3C) (*13*). We then determined whether there was an increase in the number of IFN-γ–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 enzyme-linked immunospot (ELISPOT) analysis (Fig. 3D). We found that *Aire*$^{o/o}$ mice had a statistically significant increase in the number of IFN-γ–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 whether 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, 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. The mice were killed and their organs were 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 the BALB/c major histocompatibility complex 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 IFN-γ–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 [^{3}H]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 killed 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. 5, C and D) and had a similar appearance. Our results show that the adoptive transfer of activated

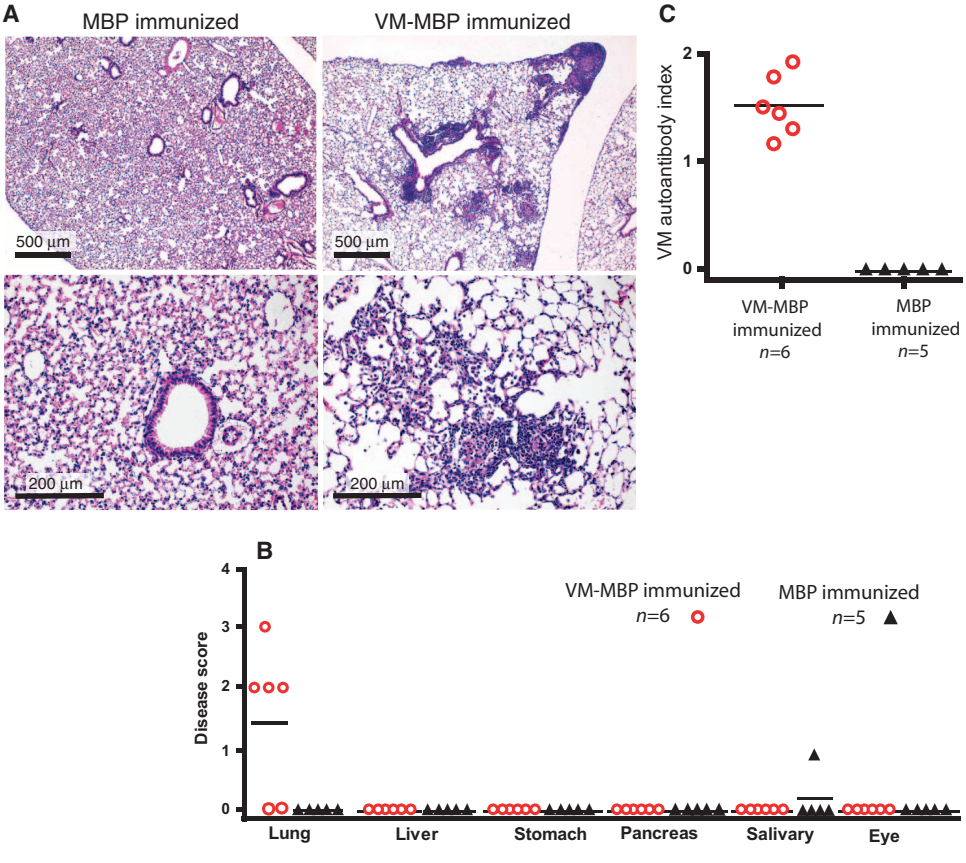


Fig. 4. Induction of lung-specific disease by breaking tolerance to VM in wild-type mice. (**A**) H&E of lungs from BALB/c WT mice immunized with VM-MBP or MBP. (**B**) Four of six mice immunized with VM-MBP exhibited lung disease, scored as shown. Mononuclear cell infiltrates were limited to the lung, except in 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.

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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 support 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 and 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 National Center for Biotechnology Information 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 (Fig. 6, D and E) (*25*). The PLUNC proteins have not previously been implicated in autoimmunity, but because their domain structure and expression pattern are similar to those of VM, we tested our APS1 patient serum against LPLUNC1, the family member with the highest concentrations of gene expression in the lung, particularly 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. 6, G and H). Our data show 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

We have shown that a loss of immune tolerance to a lung self antigen results in spontaneous ILD. We used a relatively unbiased approach to identify the lung protein VM 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

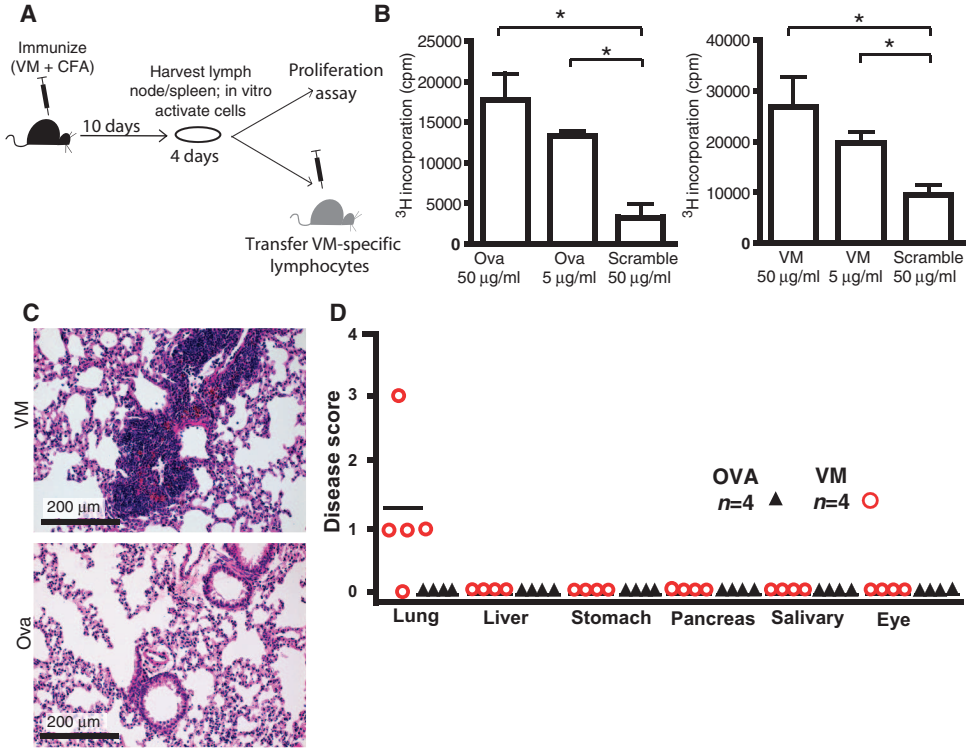


Fig. 5. Lung-specific disease after adoptive transfer of VM-specific T cells. (**A**) Protocol for adoptive transfer begins with immunization of BALB/c WT mice with VM or Ova peptide. Ten days later, lymph node and spleen cells are activated in vitro with respective peptides. Activated cells are analyzed in a proliferation assay or transferred into BALB/c SCID mice. (**B**) Representative [^{3}H]thymidine incorporation assay in cells harvested from immunized mice. Each condition was 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 killed 4 to 6 weeks after transfer and organs were 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 of antigen-specific cells.

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of ILD. Our results show how an immune response against a lung antigen can cause ILD both in the setting of multiorgan 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 include 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 ILD. 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 the lung resulted in a bronchiolitis and cellular interstitial pneumonia in *Aire*$^{o/o}$ mice that was similar to the lung disease in our APS1 patient and in other such APS1 patients described in the literature (*12*). 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

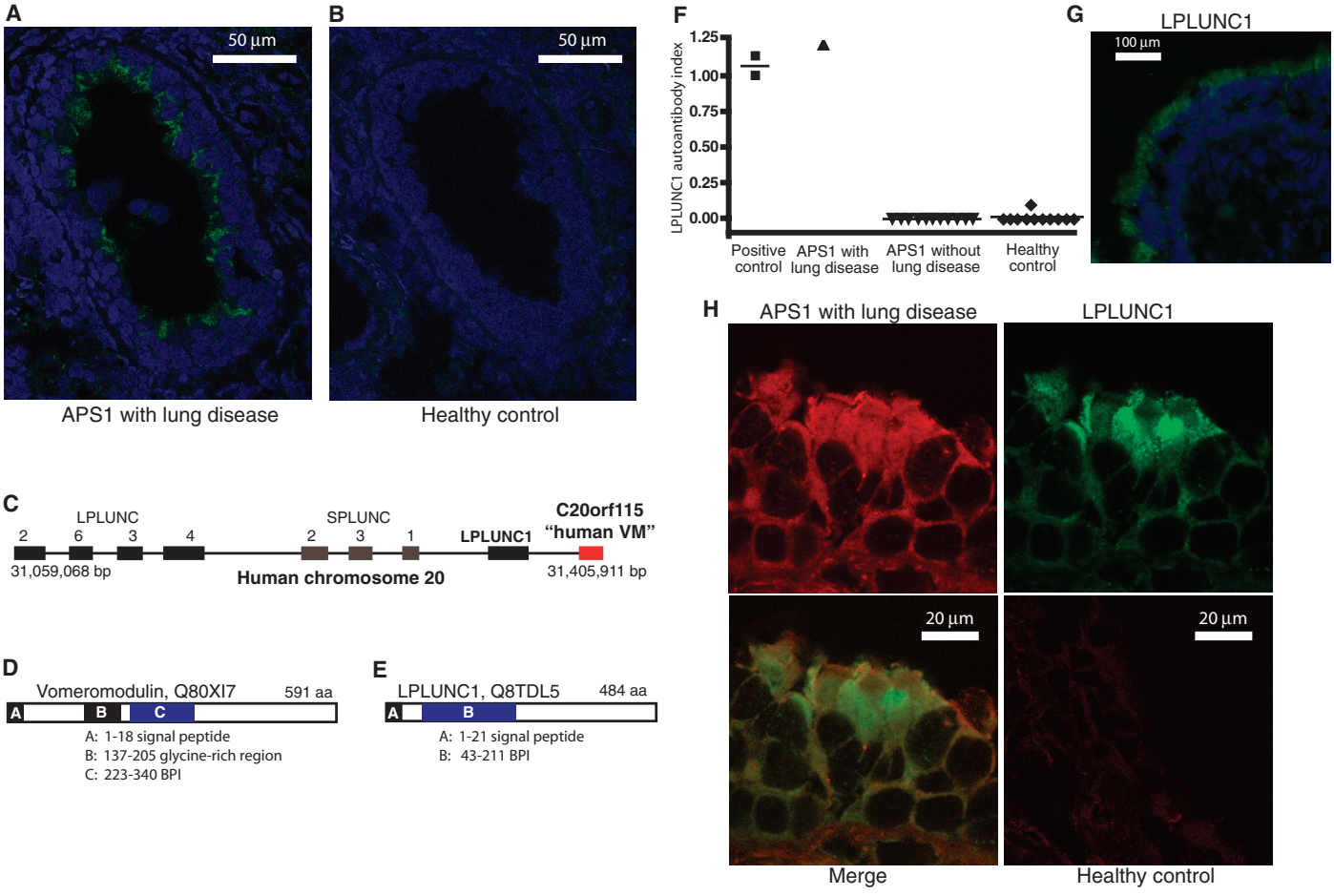


Fig. 6. Autoreactivity to a human bronchial epithelial protein, LPLUNC1, in a patient with APS1 and lung disease. (**A** and **B**) Immunofluorescence stain of normal frozen human lung with serum from an APS1 patient with lung disease (A) or a normal healthy patient (B). Green, serum staining; blue, 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 VM 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 antibodies to human LPLUNC1 were run. Shown are representative results from two 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) shows colocalization (bottom left) on the bronchiolar epithelium. A similar lung section stained with healthy patient serum does not demonstrate autoreactivity.

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been difficult to accomplish in patients. For instance, the airway-centered infiltrates appear to precede interstitial pneumonia in *Aire*$^{o/o}$ mice, which suggests that a similar temporal pattern may occur in ILD patients because 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 previous study that loss of the Aire-regulated eye autoantigen, IRBP (interphotoreceptor retinoid-binding protein), in the thymus is sufficient to induce a T cell–mediated autoimmune uveitis (*17*). Another study identified the Aire-dependent protein SVS2 (seminal vesicle secretory protein 2) 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*). Note also 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 may determine the propensity of APS1 patients and other patients with autoimmune disease to develop ILD.

There are no studies implicating VM in autoimmunity, and much remains unknown about the protein and its function. VM 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 that 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 to be important for innate immune responses (*34*, *35*). Recombinant BPI peptides may exhibit antifungal 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, at present, not 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 used 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) indicate 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 ILDs. 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

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Materials and Methods
Fig. S1. Pulmonary disease in *Aire*$^{o/o}$ mice and an APS1 patient.
Fig. S2. Patient characteristics of an APS1 patient.
Fig. S3. Characterization of lung lymphocytes in *Aire*$^{o/o}$ mice.
Fig. S4. Immunoblot of vomeromodulin after treatment with *N*-glycosidase.
Fig. S5. ELISPOT of T cells in *Aire*$^{o/o}$ mice.
Fig. S6. KCNRG autoantibody assay in the APS1 patient with lung disease.
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37. Acknowledgments: We thank J. Bluestone, A. Abbas, and H. Chapman for helpful discussions; F. Margolis for VM antisera; G. Eisenbarth and L. Yu for assistance with autoantibody assays; and M. Eckart and R. C. Winant at Stanford for mass mapping analysis.
Funding: NIH grant K08HL095659 and American Thoracic Society Fellows Career Development Award (A.K.S.); NIH grants DK59958, AI035297, and EY016408; Burroughs Wellcome Fund; and Sandler Foundation (M.S.A.); NIH grant R01CA136753A01 (Y.H. and L.F.); and Canadian Institutes of Health Research, Eli Lilly, and Canadian Foundation for HIV Research (E.B.S.).
Author contributions: A.K.S. designed and performed the experiments and wrote the manuscript; M.S.A. provided funding, designed the experiments, and wrote the manuscript; J.D., C.L.T., and K.J. provided data and helped prepare figures; C.S.O. and E.B.S. provided clinical expertise and clinical samples; K.D.J. provided histopathologic interpretation of tissue specimens; and Y.H. and L.F. provided data.
Competing interests: The authors declare no competing interests.
Accession numbers: Vomeromodulin cDNA sequence is GenBank NM_001025574.1.

Submitted 21 July 2009
Accepted 11 November 2009
Published 2 December 2009
10.1126/scitranslmed.3000284

Citation: A. K. Shum, J. DeVoss, C. L. Tan, Y. Hou, K. Johannes, C. S. O'Gorman, K. D. Jones, E. B. Sochett, L. Fong, M. S. Anderson, Identification of an autoantigen demonstrates a link between interstitial lung disease and a defect in central tolerance. *Sci. Transl. Med.* **1**, 9ra20 (2009).

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