# BPIFB1 IS A LUNG-SPECIFIC AUTOANTIGEN ASSOCIATED WITH INTERSTITIAL LUNG DISEASE[**]

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## Abstract

Interstitial lung disease (ILD) is a complex and heterogeneous disorder that is often associated with autoimmune syndromes (1). Despite the connection between ILD and autoimmunity, it remains unclear whether ILD can develop from an autoimmune response that specifically targets the lung parenchyma. Here, we utilized a severe form of autoimmune disease, Autoimmune Polyglandular Syndrome Type 1 (APS1), to establish a strong link between an autoimmune response to the lung-specific protein BPIFB1 and clinical ILD. Screening of a large cohort of APS1 patients revealed autoantibodies to BPIFB1 in 9.6% of APS1 subjects overall and in 100% of APS1 subjects with ILD. Further investigation of ILD outside the APS1 disorder revealed

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BPIFB1 autoantibodies specifically present in 14.6% of patients with connective tissue disease-associated ILD and in 12.0% of patients with idiopathic ILD. Utilizing the animal model for APS1 to examine the mechanism of ILD pathogenesis, we found that *Aire*$^{-/-}$ mice harbor autoantibodies to a similar lung antigen named BPIFB9 that are a marker for ILD, and determined that a defect in thymic tolerance is responsible for the production of BPIFB9 autoantibodies and the development of ILD. Importantly, we also found that immunoreactivity targeting BPIFB1 independent of a defect in Aire also leads to ILD, consistent with our discovery of BPIFB1 autoantibodies in non-APS1 patients. Overall, our results demonstrate that autoimmunity targeting the lung-specific antigen BPIFB1 may be important to the pathogenesis of ILD in patients with APS1 and in subsets of patients with non-APS1 ILD, demonstrating the role of lung-specific autoimmunity in the genesis of ILD.

## Introduction

Interstitial lung disease (ILD) encompasses a heterogeneous group of disorders characterized by chronic inflammation and fibrosis within the connective tissue of the lung. The onset of ILD has been correlated with many exposures and well-characterized diseases but the primary mechanisms that trigger ILD remain elusive. Increasing evidence suggests a potential role of autoimmunity in ILD pathogenesis (2–4). ILD can develop in nearly all autoimmune connective tissue diseases, including common disorders like rheumatoid arthritis (1, 4). Due to the lack of specific markers to diagnose lung autoimmunity, it remains unclear whether patients with idiopathic ILD may have a component of lung autoimmunity, as select patients with idiopathic ILD also share identical histopathology to ILD patients with defined autoimmune syndromes (2, 3, 5, 6). Prior studies have not identified an autoantigen clearly involved in the pathophysiology of ILD, and none of the putative antigens associated with ILD show lung-specific expression, raising the question whether such autoantibodies arise as secondary phenomena or through epitope spreading (7–9). Thus, despite evidence implicating autoimmunity in ILD, studies have yet to identify a pathogenic mechanism whereby an autoimmune response specifically targets the lung within ILD patients.

Insights from rare extremes of disease can be applied to understand more common disorders; for example, the discovery of the type 1 diabetes-associated autoantigen GAD65 arose from studying patients with a rare, severe autoimmune disease termed Stiff Person Syndrome (10). We hypothesized that this approach could be used to identify lung autoantigens in ILD by studying subjects with the rare monogenic disorder, Autoimmune Polyglandular Syndrome Type 1 (APS1). APS1 patients develop multiple organ-specific autoimmune diseases that are associated with autoantibodies to established organ-specific antigens (11, 12). Recently, ILD has been recognized as an important manifestation of APS1 and is characterized by prominent bronchiolar inflammation (13, 14), similar to ILD commonly seen in systemic autoimmune disorders (1, 15). Importantly, APS1 arises from defects in the *Autoimmune regulator* (*AIRE*) gene, a key modulator of central immune tolerance with a known mechanism and well-defined animal model (16). Thus, this model represents an attractive system of organ-specific autoimmunity to examine lung-specific autoimmunity in ILD.

## Results

### Autoantibodies to the BPIFB1 lung antigen in APS1 patients with ILD

We first noted autoantibodies to BPIFB1 (bactericidal/permeability-increasing fold-containing B1) protein in an APS1 patient with ILD (14). To determine if BPIFB1 autoantibodies are broadly specific for ILD in subjects with APS1, we tested a large APS1

cohort for BPIFB1 autoantibodies using a radioligand-binding assay (RLBA). A limited number of APS1 subjects or controls had values near the assay cut-off consistent with non-specific binding but 10/104 (9.6%) APS1 subjects had markedly elevated serum autoantibodies against BPIFB1 (Fig. 1). Review of the available clinical data revealed 6 APS1 patients with clinically significant pulmonary disease verified in pulmonary function tests (PFT) or chest radiographs (Table 1). Remarkably, 100% of APS1 patients with ILD had high BPIFB1 autoantibody titers, and all available biopsies revealed ILD characterized by bronchiolar inflammation in these patients (5/6). None of the other APS1 patients tested had abnormal PFTs or chest radiographs at the time of serum sampling. Based on these results, the sensitivity and specificity of our assay, using an ROC cutoff value of 16, were 100% (CI 54.07–100%) and 94.9% (88.49–98.32%), respectively (Fig. S1) and the area under the curve (AUC) in the receiver operating characteristics analysis was 0.987 (95% CI 0.966 to 1.01; P<0.0001). These data suggest that the BPIFB1 autoantibody assay is a highly sensitive and specific test for detecting pulmonary disease that has been confirmed in PFTs or chest radiographs.

Having shown that BPIFB1 autoantibodies are highly sensitive and specific for ILD in APS1, we next asked whether BPIFB1 exhibits a tissue restricted expression pattern similar to other antigens in the Aire model. We investigated the human tissue expression of BPIFB1 by quantitative real-time PCR. High levels of BPIFB1 transcripts were limited to the lung and low levels were found in multiple organs including the thymus, the location outside of the lung with the highest tissue expression (Fig. S2). Importantly, this pattern of both tissue-specific and thymic expression mirrors other organ-specific antigens identified in the Aire-deficient model that have critical roles in the pathogenesis of organ autoimmunity (14, 17–20). Taken together, our results demonstrate that BPIFB1 is a lung-specific autoantigen in APS1 patients with interstitial lung disease.

## Autoantibodies to BPIFB1 in ILD patients without APS1

We next hypothesized that that the BPIFB1 antigen may be targeted in a subset of ILD patients outside of the APS1 disorder, especially in the setting of systemic autoimmunity. Screening of a heterogeneous cohort of patients with connective tissue disease-associated ILD (Fig. S3) revealed that 14.6%, (7/48) had elevated BPIFB1 autoantibodies (Fig. 2, Table S1). Given that subclinical or occult lung-restricted autoimmunity could play a role in patients without other known etiologies for ILD, we extended screening to a large, heterogeneous cohort of subjects with idiopathic ILD (Fig. S4). We found that 12.0% (13/108) of such patients harbored BPIFB1 autoantibodies (Fig. 2, Table S2), whereas no BPIFB1 autoantibodies were detected in non-ILD lung disease controls matched for age and race to all of the non-APS1 BPIFB1-positive subjects (Fig. 2, Fig. S5). Having identified a subset of idiopathic and connective tissue disease-associated ILD (CTD-ILD) patients that were positive in the BPIFB1 assay, we next tested them specifically for autoantibodies to IFNα. The IFNα autoantibody assay is a highly sensitive and specific test to diagnose APS1 (Fig. S6B), including cases of undetected or subclinical disease (21–23). We determined that 100% of APS1 patients had circulating IFNα autoantibodies, whereas none of the CTD-ILD or idiopathic ILD patients had a positive IFNα titer, confirming that these subjects did not have APS1 disease (Fig. S6A). Finally, in order to verify that BPIFB1 autoantibodies are not broadly associated with autoimmune disorders or autoimmune inflammation, we tested subjects with type 1 diabetes, a disease characterized by multiple circulating autoantibodies without lung pathology, and found that none of these patients were positive in the assay (Fig 2). Taken together, these results suggest that BPIFB1 autoantibodies are a specific marker for lung autoimmunity.

## Validation of the BPIFB1 radioligand binding assay

To characterize the tissue specificity of serum BPIFB1 autoantibodies, we performed indirect immunofluorescence staining of fresh frozen lung sections (Fig. 3A). Staining with a commercial BPIFB1 antibody revealed reactivity to the bronchiolar epithelium. Strikingly similar staining was seen using the serum from either an APS1 ILD patient or a non-APS1 ILD patient with BPIFB1 autoantibodies. To validate this result, we performed a competition reaction in which the serum used for staining was pre-absorbed with commercial unlabeled recombinant BPIFB1 and then tested in the RLBA. We could successfully compete out specific binding of BPIFB1 serum autoantibodies using the unlabeled recombinant BPIFB1 protein (Fig. 3B). Finally, to further confirm that the autoantibody response in our RLBA was specific for the BPIFB1 protein, we also performed a sequential immunoprecipitation with radiolabeled BPIFB1. Initial immunoprecipitations were performed using serum samples with and without BPIFB1 autoantibodies. The second and final immunoprecipitation was performed with a commercial anti-human BPIFB1 antibody to capture any remaining antigen. Only samples from patients positive for BPIFB1 autoantibodies on RLBA were able to deplete out the radiolabeled BPIFB1 protein (Fig. 3C). The identification of BPIFB1 autoantibodies in ILD subjects suggests that the bronchiolar epithelium may be a critical target for autoimmune lung reactions and provides further evidence that terminal bronchioles may be an important site in the pathogenesis of ILD and pulmonary fibrosis (24).

## Autoantibodies to BPIFB9 as a marker for ILD in Aire-deficient mice

Autoantibodies frequently arise from recognition of cognate antigens with corresponding T-cell specificity. As such, they can directly mediate pathogenic effects in some diseases (25) or arise secondary to a T cell driven response (26). The contribution of antigen-specific B and T cell immunity can be difficult to assess in human subjects (27). Thus, to better examine the autoimmune mechanism of ILD induction, we turned to the $Aire^{-/-}$ mouse model of APS1, which manifests multi-organ autoimmunity and lung disease as in APS1 patients (14). Significantly, we recently identified the lung self-antigen BPIFB9, a murine BPIFB family protein with high similarity to BPIFB1, in $Aire^{-/-}$ mice with lung autoimmunity (14). First, to establish if BPIFB9 autoantibodies in our mouse model also act as a marker for ILD, as demonstrated in our human cohorts, we assessed $Aire^{-/-}$ animals by both RLBA and lung histology scores (Fig. 4A). Nearly all $Aire^{-/-}$ mice with histologic lung disease (24/27, 89%) had BPIFB9 autoantibodies while no autoantibodies were detected in wild-type controls. Moreover, the mean autoantibody index of mice with severe disease (score=3 mean index=95.03) was significantly elevated over $Aire^{-/-}$ mice without histologic disease (score=0, mean index=10.5, P=0.0002) or mild disease (score=1, mean index=48.3, P=0.0185). Given that BPIFB9 autoantibody titers appear to directly correlate with disease severity, we next sought to evaluate whether BPIFB9-specific autoantibodies are sufficient to cause disease. We generated serum with elevated BPIFB9 autoantibodies by immunizing $Aire^{-/-}$ mice with the BPIFB9 protein. We confirmed that the serum isolated from these mice contained markedly elevated BPIFB9 autoantibodies (Fig. S7A) and then intravenously injected 50uL of this serum or wild-type control serum every other day into 2 separate cohorts of wild-type mice over a period of 10 days. One week after the final serum transfer all of the mice were sacrificed and analyzed for disease. None of the mice in either group developed lung disease (Fig. S7B). Thus, while the BPIFB9 autoantibody index correlates with disease severity and may serve as a marker of murine ILD, BPIFB9 autoantibodies do not appear to be sufficient for induction of ILD.

### Induction of ILD by BPIFB9-specific CD4$^+$ T cells

Given the important role CD4$^+$ T cells play in orchestrating autoimmune diseases and the critical role of Aire in negatively selecting autoreactive T cells (16, 28), we next sought to determine if autoreactive CD4$^+$ T cells specific for the BPIFB9 protein could induce ILD. *Aire*$^{-/-}$ mice were immunized with recombinant BPIFB9 or a control protein (maltose binding protein tag) to expand a population of antigen-specific T cells. CD4$^+$ T cells were isolated from lymphoid organs 10 days post-immunization and confirmed by Enzyme-linked immunosorbent spot (Elispot) for the presence of an expanded population of antigen-specific cells (Fig. 4B), prior to adoptive transfer (4x10$^6$ – 10x10$^6$ CD4$^+$ T cells/recipient) into immunodeficient mice. Recipients were analyzed 4–8 weeks post-transfer for lung pathology. Nearly all mice receiving BPIFB9-specific cells (7/9) demonstrated CD4 T-cell infiltrates similar to the spontaneous ILD in *Aire*$^{-/-}$ mice (Fig. 4C). Overall the disease in BPIFB9-recipients was significantly greater than in mice receiving MBP-specific cells (Fig. 4C–D), indicating that BPIFB9-specific T cells are sufficient to cause ILD. Additionally, because we detected BPIFB1 autoantibodies in some patients with lung fibrosis, we assessed whether the murine autoimmune ILD seen in *Aire*$^{-/-}$ mice can evolve into other pathological features of ILD, including interstitial pneumonia and fibrosis. Aged animals at 23 weeks and 45 weeks developed spontaneous interstitial pneumonia and significant pulmonary fibrosis (Fig. 4E), particularly at 45 weeks, indicating these pathological features of ILD can develop downstream of an autoimmune response to a lung self-antigen. Importantly, our studies in the murine model of human APS1 ILD confirm that lung antigen-specific T cell responses can induce ILD and recapitulate key features of lung-specific autoimmunity seen in human subjects with ILD.

### Role of thymic tolerance in ILD

We next sought to investigate whether central tolerance induction to lung proteins expressed in the thymus has a potential role in ILD. Having previously demonstrated that thymic BPIFB9 expression is Aire-dependent (14), we first confirmed that this expression pattern maps specifically to medullary thymic epithelial cells (mTEC) in *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mice given the essential function mTECs have in maintaining central immune tolerance (Fig. 5A). In order to test whether a loss of lung-antigen expression in the thymus leads to ILD, we performed a thymic graft experiment in mice by transplanting donor thymi from BALB/c *Aire*$^{-/-}$ and *Aire*$^{+/-}$ newborn mice into athymic BALB/c nude mice (n=4 for each group). Sera from recipient mice were analyzed 10 weeks post-transplant specifically for BPIFB9 autoantibodies since we demonstrated this is a marker for ILD in our model. All of the recipients that received *Aire*$^{-/-}$ thymi developed BPIFB9 autoantibodies (mean index=74.08) and histologic evidence of lung disease with lymphocytic infiltrates of T and B cells (Fig. 5B–D), whereas none of the *Aire*$^{+/-}$ thymi recipients had detectable autoantibody levels (mean index=0.1892, *P=0.0286*). Taken together, these results demonstrate the importance of thymic lung antigen expression to ILD in the Aire model and suggest a potential role that central tolerance to lung antigens such as BPIFB1 has in human ILD pathogenesis.

### Autoimmune targeting of BPIFB1 in ILD

Having established a strong link between BPIFB1 autoreactivity and human ILD, we next sought to demonstrate that a break in tolerance to BPIFB1 could lead to autoimmune ILD. Because BPIFB1 autoantibodies are found in non-APS1 ILD patients, we wished to test for autoimmunity to BPIFB1 and lung disease independent of a defect in Aire. We obtained *Bpifb1*$^{-/-}$ mice because immune cells in these animals may in theory be unable to unable to develop self-tolerance to BPIFB1 (29). Indeed, *Bpifb1*$^{-/-}$ mice immunized with recombinant BPIFB1 protein developed robust BPIFB1-specific B and T cell responses that

were not seen in mice immunized with a control antigen (Figs 6A–B). Lymphocytes were harvested from the spleens of these mice and 2.5 x10$^7$ cells were adoptively transferred into BPIFB1-sufficient $Rag2^{-/-}$ animals. Mice receiving BPIFB1-specific immune cells collected from $Bpifb1^{-/-}$ mice immunized with BPIFB1 protein developed lung disease indistinguishable from the lung infiltrates in $Aire^{-/-}$ mice and APS1 patients, whereas mice receiving cells from wild-type immunized littermates did not develop any disease (Fig. 6D). Thus, inducing a break in immune tolerance to BPIFB1 allows for the generation of BPIFB1-reactive cells that are capable of inducing lung-specific ILD and robust BPIFB1-specific autoantibodies. Taken together, our results strongly suggest that immunoreactivity to BPIFB1 leads to autoimmune lung disease in a subset of patients, both as a result of a negative selection defect mediated by Aire and in other ILD settings in which a loss of self-tolerance occurs.

## Discussion

In summary, we show that autoantibodies to the lung self-antigen BPIFB1 are found in diverse subjects with ILD and may serve as a marker of lung-specific autoimmune disease. In our study, 100% of patients with APS1 and clinically verified ILD demonstrate BPIFB1 autoantibodies, confirming a strong correlation between ILD and our marker. By extending these findings from APS1, a model of organ-specific autoimmunity, to patients with systemic autoimmunity in CTD-ILD, we find that BPIFB1 is also a targeted lung antigen outside of APS1 disease. Moreover, the presence of BPIFB1 autoantibodies in idiopathic ILD is consistent with a marker of lung-specific autoimmunity rather than a general marker associated with autoimmune inflammation. Our demonstration in mice that BPIFB1-specific immune responses leads to ILD, provides substantial evidence that autoreactivity targeting BPIFB1 is pathogenic in a subset of human ILD patients.

By translating clinical studies in humans to immunologic studies in the murine model, we are able to demonstrate a lung-specific mechanism of disease pathogenesis. Though earlier investigations have suggested a role for autoimmune injury in ILD pathogenesis, they have not established a mechanistic link to lung-specific autoimmunity. For example, ILD patients harbor autoantibodies to human lung and lymphocytes that proliferate in response to autologous human lung extracts, and studies in limited cohorts have described autoantibodies to annexin-1 and cytoskeletal proteins, including antigens reported in other inflammatory disease (7–9). Given the wide tissue distribution of these putative targets, such antigens may represent less dominant epitopes that arise during systemic inflammation (30, 31). In contrast, our lung-specific marker is significantly elevated in a subset of a large ILD cohort without significant elevation in over 350 control subjects and the corresponding marker tracks with disease severity in our animal model of ILD. Moving beyond disease correlation, we show directly in our murine model how the loss of immune tolerance to a lung self-antigen may provoke a lung-specific autoimmune response by demonstrating that a lack of BPIFB9 expression in the thymus led to the induction of BPIFB9 autoantibodies and lung infiltrates comprised of T and B cells. Furthermore, we found that the adoptive transfer of lung-specific, BPIFB9-specific CD4 T cells induces ILD. Finally, in order to confirm the pathogenicity of the BPIFB1 antigen and to evaluate the role of autoimmune targeting of BPIFB1 in non-APS1 patients, we demonstrated that immunoreactivity to the BPIFB1 lung antigen leads to ILD independent of a defect in Aire. Taken together, these data support the role of a break in immune tolerance to BPIFB1 in ILD and furthermore suggests a potential link of failed thymic tolerance to ILD pathogenesis beyond the APS1 disorder.

Several important questions are raised by the results of our study. First, we find that antibody-positive individuals can manifest a range of lung pathology. We demonstrated in mice that immune targeting of BPIFB1 is sufficient to cause ILD, though it remains unclear

whether BPIFB1 is the dominant antigen in disease pathogenesis. BPIFB1 may represent one of several lung autoantigens associated with ILD; for example, another bronchiolar antigen, KCNRG (Potassium channel regulator), has been identified in APS1 patients (13), and it is possible that peripheral lung proteins are also targeted as lung disease propagates. Such a temporal pattern would be consistent with our observation in mice that the ILD is initiated at the bronchiolar epithelium and subsequently spreads distally to cause pathologic changes in the peripheral parenchyma (14). Moving forward, it will be important to determine if autoreactivity to other lung antigens can likewise be mechanistically linked to ILD and found in other subsets of ILD patients. Another question is why do we see ILD both in combination with a range of autoimmune disorders as well as in patients with idiopathic forms of the disease? Because our work supports a model where loss of tolerance to one or more lung-specific autoantigens leads to lung-specific injury and autoimmunity with resultant ILD, it is possible that different paths of tolerance breakdown progress to a common endpoint of ILD. Patients without mutations in *AIRE* may have other, less severe defects in immune tolerance that also predispose to autoimmunity and in the setting of such tolerance defects, certain forms of lung injury or stress in the pulmonary epithelium may go on to produce ILD (32, 33). Finally, the significance of autoantibodies in disease-free individuals requires further study. Prospective study of samples from at-risk patients will be important to determine if BPIFB1 autoantibodies predict development of ILD, consistent with other autoimmune diseases where positive autoantibodies precede clinical disease (34, 35). Notably, monitoring of APS1 patients positive for BPIFB1 autoantibodies without current lung disease (Table 1) may be revealing.

Our findings also have several other important clinical implications. First, the BPIFB1 assay may identify ILD patients with lung-specific autoimmunity that may benefit from immunomodulatory treatments. Second, identifying critical target antigens like BPIFB1 will enable the study of key effector mechanisms of autoimmune damage allowing for development of novel, targeted immunotherapies that obviate generalized immunosuppression (36, 37). Finally, the study of BPIFB1 antigen may lead to development of additional diagnostic tools to measure BPIFB1-specific T cells in clinical samples such as bronchoalveolar fluid or peripheral blood (27). Such immune cells may be critical mediators of ILD and their measurement may help predict a clinical response. The results here combine the examination of basic tolerance mechanisms in a mouse model with insights gained from a rare human autoimmune syndrome to allow a broader understanding of potential targets of disease in a subset of ILD patients. Looking forward, continuing to follow such an approach may continue to provide new insights into ILD pathogenesis, prognosis, and ultimately its treatment.

## Materials and Methods

### APS1 subjects, ILD subjects and controls

All subjects in the study had informed written consent. The study protocol was reviewed and approved by the institutional review board at UCSF and ethics committee at Uppsala University. Sera from 27 non-ILD lung disease controls, 48 patients with connective-tissue associated ILD and 108 patients with idiopathic ILD were obtained from the UCSF Interstitial Lung Disease database, a longitudinal cohort study at UCSF (38). Subject diagnosis was made based on a multidisciplinary review in the UCSF ILD clinic. Sera from 104 subjects with confirmed APS1 and control subject sera in Figure 1 comprising of 126 healthy blood donors, 51 patients with asthma and 51 patients with chronic obstructive pulmonary disease (COPD) are from an IRB approved cohort study at Uppsala University (12, 13). The diagnosis of APS1 was based on presence of at least 2 of 3 major clinical components of APS1 (Addison's disease, hypoparathyroidism, and chronic mucocutaneous

candidiasis). The following diagnostic criteria were used: mucocutaneous candidiasis (candidal infections in the oral mucosa, skin or nails for >3 months); hypoparathyroidism [subnormal plasma calcium concentration (<2.15 mmol/L) and supranormal plasma phosphate concentration together with normal or low PTH concentrations, and normal renal function]; Addison's disease (subnormal serum cortisol together with elevated plasma ACTH concentrations or failure to reach s-cortisol of 550 nmol/L at 30 or 60 min of an ACTH stimulation test) [the majority of the patients diagnosed with Addison's disease also displayed specific 21-hydroxylase autoantibodies; the majority of the patients were also demonstrated to have typical mutations in the Aire gene. Other control sera shown in Figure 2 comprising 42 Type 1 diabetes subjects, 35 Type 2 diabetes subjects and 46 healthy subjects were obtained from banked samples in an IRB approved cohort study at the UCSF Diabetes Center.

### Generation of $^{35}$S-Radiolabeled BPIFB1, BPIFB9 and IFNα radioligand binding autoantibody assays (RLBA)

Full-length cDNAs for human BPIFB1 or IFNα and mouse BPIFB9 or BPIFB1 were used as templates for *in vitro* transcription and translation and labeled with $^{35}$S-methionine using the TNT system kit (Promega). Radiolabeled proteins were immunoprecipitated in duplicate or triplicate with serum or control antibodies in 96-well PVDF filtration plates (Millipore). In each well, 20,000 counts per minute (cpm) of $^{35}$S-proteins and 2.5μl serum were used for immunoprecipitation. The radioactivity of the immunoprecipitated material was quantified with the use of a liquid scintillation counter. For each assay, serum from an APS1 patient with ILD and a healthy control (BPIFB1 or IFNα); serum from an *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mouse (BPIFB9); or serum from a *Bpifb1*$^{+/+}$ and *Bpifb1*$^{-/-}$ mouse (BPIFB1) served as positive and negative standards, respectively. The autoantibody index was calculated as follows: [cpm in the unknown sample –cpm in the negative standard] ÷ [cpm in the positive standard– cpm in the negative standard] x 100). RLBAs were performed in 2 independent laboratories using the same antigen construct. The upper limit of the normal range for each assay was defined as the mean value obtained for the healthy control patients tested in each lab plus 4 SD. In the competition assay, the sample was pre-incubated with 2.5μg of BSA or recombinant BPIFB1 protein (Abnova H00092747-P01) before running through the RLBA as described above.

### Sequential Immunoprecipitation

Recombinant $^{35}$S-radiolabeled protein (150,000 cpm) was precipitated with a commercial anti-BPIFB1 antibody (Abnova #H00092747-B01P) as a control or 2.5μl of serum from each patient. Antibody-antigen complexes were captured on protein-A Sepharose beads and then removed by centrifugation. The remaining supernatant was subjected to a second round of immunoprecipitation using the same serum sample. The third and final immunoprecipitation of protein was performed with the commercial anti-BPIFB1 antibody and the immunoprecipitate was analyzed by SDS-PAGE, followed by autoradiography. The signal intensities were quantified using Kodak Molecular Imaging Software.

### Recombinant BPIFB1 and BPIFB9 proteins

Recombinant BPIFB9 protein was generated as previously described (14). Mouse BPIFB1 protein was generated according to the same methods. The primers used for subcloning mouse BPIFB1 into the expression vector were: forward 5′-TTAGGTACCAATGGCCGGCCCGTG-3′; reverse 5′-TAACCTGCAGGTCACTGGGAGACAG-3′.

### Indirect Immunofluorescence Staining

Frozen sections (8μm) of bovine lung tissue were fixed and blocked in PBS + 1% bovine serum albumin + 3% serum from same species as the secondary antibody, overnight at 4°C. Primary incubation with subject sera (1:1000) was for an hour followed by a donkey anti-human Dylight 594 (Jackson Immunoresearch) secondary antibody (1:1000) for 20 minutes. An anti-BPIFB1 antibody (Abnova, #H00092747-B01P) (1:50) was incubated for an hour, followed by a goat anti-mouse Dylight 594 secondary (Jackson Immunoresearch) (1:1000) for an hour. Images were obtained at 20X magnification on a confocal microscope (SP5, Leica).

### Immunostaining

Immune cell subtypes were visualized by immunohistochemistry using antibodies specific for CD4 (BD Biosciences), CD3 (Dako), and B220 (SoutherBiotech) and Elite ABC reagent followed by a DAB staining kit (Vector Laboratories) on 10-micron frozen or 5-micron paraffin-embedded sections of lungs. Images were obtained using an AxioCam with AxioVision software (both from Carl Zeiss MicroImaging, Inc.) at 5X and 10X objective on a brightfield microscope (Carl Zeiss).

### Mice

$Aire^{-/-}$ mice were generated as described (14). Bpifb1$^{-/-}$ mice were generated by Lexicon Pharmaceuticals and obtained from the Mutant Mouse Regional Resource Center (Stock: 032698-UCD)(39). Rag2$^{-/-}$ mice used in the BPIFB1 adoptive transfer experiments were purchased from Taconic (RAGN12). All mice were housed in a pathogen-free barrier facility at UCSF and all procedures were approved by the UCSF Institutional Animal Care Committee and Veterinary Services, and adhere to the NIH Guide for the Care and Use of Laboratory Animals. Organs from mice were harvested, a portion of which was snap frozen for frozen sectioning and the remaining tissue fixed overnight in 10% formalin, embedded in paraffin and sectioned. The UCSF Mouse Pathology Core performed Masson's Trichrome and hematoxylin and eosin staining.

### Immunization and adoptive transfer of immune cells and serum

$Aire^{-/-}$ mice were immunized subcutaneously with 200μL of a 1:1 mixture of complete Freund's adjuvant (CFA) (6mg/mL) and recombinant BPIFB9 (2mg/mL) or maltose binding protein (MBP) and ten days post-immunization mice were sacrificed. The serum was collected and tested in the BPIFB9 RLBA to assess for BPIFB9 autoantibodies. Draining lymph nodes and spleens were harvested and CD4 cells were isolated by negative selection using magnetic bead separation (Stemcell technologies). 4–10x10$^6$ cells per mouse were transferred into immunodeficient mice by retro-orbital injection. Mice were sacrificed 4–8 weeks post-immunization, and lungs were infused with OCT and snap frozen for analysis. Fifty microliters of serum from BPIFB9-immunized mice or wild-type controls was injected every other day for a period of 10 days into wild-type mice and 7 days later the mice were sacrificed and lungs analyzed for histological evidence of disease. $Bpifb1^{-/-}$ mice were immunized intraperitoneally with 200μL of a 1:1 mixture of CFA (6mg/mL) and recombinant BPIFB1 (2mg/mL) followed by weekly subcutaneous immunizations for 3 additional weeks with recombinant protein mixed in incomplete Freund's adjuvant. Mice were bled weekly to check for BPIFB1 autoantibodies and once sacrificed 2.5 x10$^7$ splenocytes were adoptively transferred into $RAG2^{-/-}$ mice.

### Thymic transplantation

Thymi were removed from 1–2 day old BALB/c $Aire^{-/-}$ or $Aire^{+/-}$ animals and placed into culture in DMEM media supplemented with 100 U/ml penicillin, 100 mcg/ml streptomycin,

2 mM glutamine, 10% FCS, and 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 7 days to deplete bone marrow-derived cells. The thymi were washed in DMEM media without 2-deoxyguanosine for 2 h and transplanted under the kidney capsule of adult nude mice on the BALB/c background. Ten weeks after transplantation, the serum was analyzed for BPIFB9 autoantibody titer and paraffin-embedded lungs analyzed for tissue infiltration of T (CD3) and B (B220) cells as described above.

### Elispot

In brief, plates (Millipore) were coated with 2 $\mu$g/ml of anti–mouse IFN $\gamma$ mAb (BD Biosciences) and incubated overnight at 4°C. After blocking plates with complete media, $1 \times 10^6$ cells from lymphoid organs of immunized mice were plated in triplicate into wells containing media and 10$\mu$g/mL of recombinant BPIFB9, BPIFB1, ova peptide or ovalbumin protein. The plates were incubated for 36 hrs. at 37°C. At the completion of the incubation, the plates were washed thoroughly with PBS before adding 2 $\mu$g/ml of biotin-labeled IFN $\gamma$ mAb (2 $\mu$g/ml; BD Biosciences) and incubating for 2 hrs. at 4°C. After further incubation with avidin–horseradish peroxidase (1:100 dilutions; BD Biosciences) for 1 h at room temperature, the plates were developed using substrate solution (BD Biosciences) and counted.

### RNA isolation and quantitative PCR (qPCR) analysis

Total human RNAs were commercially purchased (Ambion and BioChain Institute). One microgram of total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen). QPCR was performed with human BPIFB1 (assay ID Hs00264197_m1) and GAPDH (assay ID Hs03929097_g1) Taqman gene expression assays (Applied Biosystems). BPIFB1 transcript was quantified using tracheal levels as a standard and normalized to GAPDH transcript levels in each sample. The thermal cycles of the ABI Prism 7500 Sequence Detection System (Applied Biosystems) were 95°C for 20 seconds followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

For mouse mTEC gene expression analysis, stromal cells from thymi of 5-week-old BALB/c *Aire$^{-/-}$* or *Aire$^{+/+}$* mice (n=5 for each) were isolated as previously described (19) and sorted on BD FACSAria III cytometers to obtain mTEC cells (DAPI$^-$ CD45$^-$ EpCAM$^+$ Ly51$^-$ CD11c$^-$). Total RNA from sorted mTEC cells was isolated (RNeasy Plus Microprep, QIAGEN) for cDNA synthesis (SuperScript III reverse transcriptase, Invitrogen) using oligo dT primers. QPCR for mouse BPIFB9 (assay ID #Mm00723516_m1) and GAD67 (assay ID Mm04207422_g1) were conducted as above. Expression was normalized to beta-actin (4352933E, Applied Biosystems) and quantified using a relative standard curve based on lung and thymus tissue levels.

### Statistics

P values for significance are indicated in the Figure legends and were all calculated with Prism software (GraphPad V5.0) using unpaired t-tests (Mann Whitney) with a two-tailed 95% confidence interval. Receiver operating characteristics analyses and area under the curve calculations were also performed with Prism software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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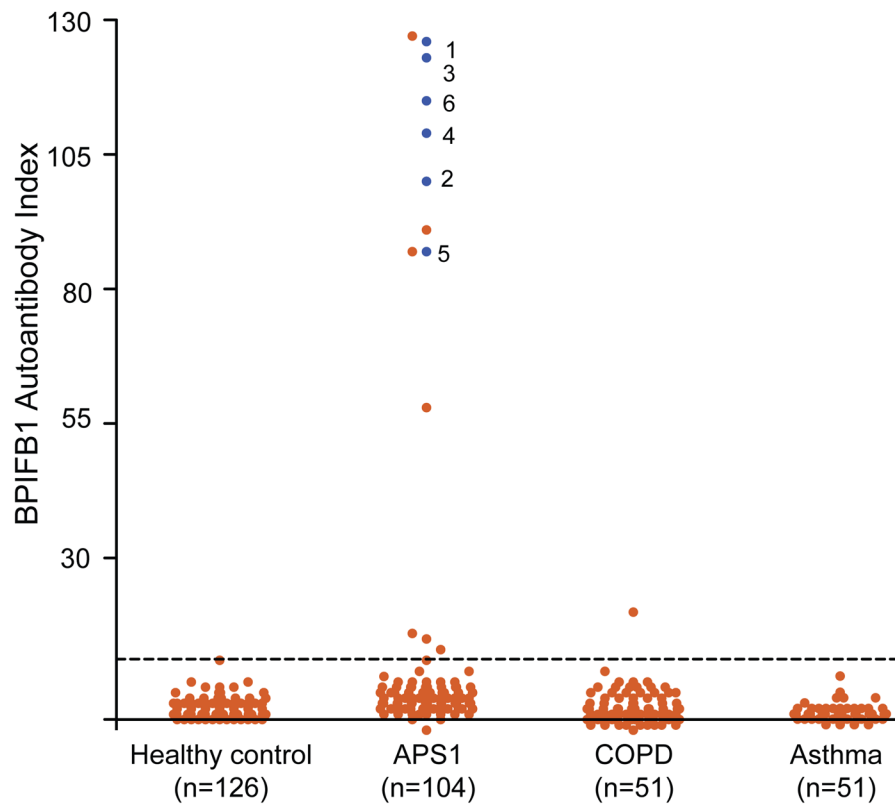
**Fig. 1. APS1 patients with ILD harbor autoantibodies to BPIFB1**

APS1 patients were screened for BPIFB1 autoantibodies by testing serum in a radioligand-binding assay (RLBA). Ten APS1 patients had elevated BPIFB1 autoantibody titers. Six patients (numbered in blue) had pulmonary disease confirmed in PFTs or radiographs (Table 1). Sera from healthy subjects and patients with COPD and asthma were run as controls. The dotted line denotes the upper limit of the normal range, defined as the average value of the healthy subjects shown + 4SD.
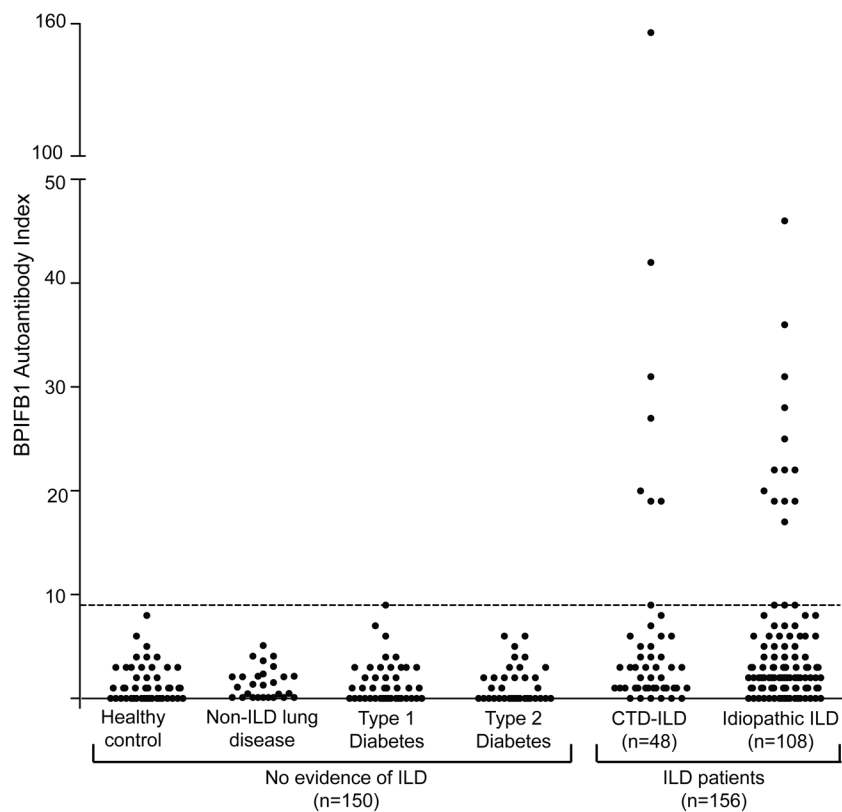
**Fig. 2. Patients with non-APS1 ILD harbor autoantibodies to BPIFB1**
Patients were screened for BPIFB1 autoantibodies by RLBA. Seven patients with connective tissue disease-associated ILD (CTD-ILD) and thirteen idiopathic ILD patients had elevated BPIFB1 autoantibody titers (Tables 2–3). Sera from healthy subjects (n=46) and patients with Type 1 (n=42) and Type 2 (n= 35) diabetics and non-ILD lung disease (n=27) were run as controls. The dotted line denotes the upper limit of the normal range, defined as the average value of the healthy subjects shown + 4SD.
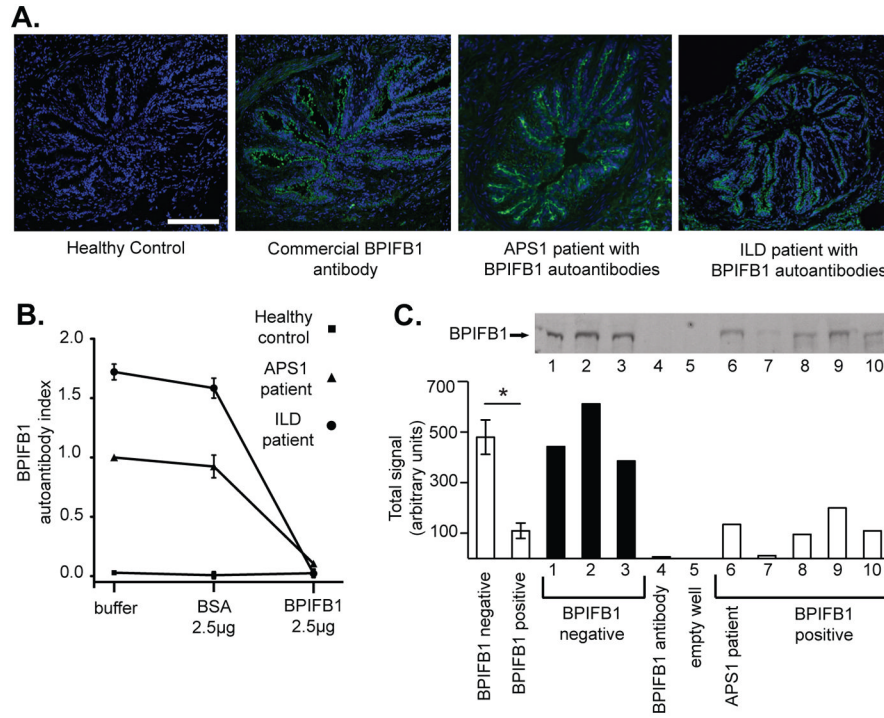
**Fig. 3. Validation of the BPIFB1 radioligand binding assay**

(A) Indirect immunofluorescence staining of frozen lung sections. Stainings (from left to right) are shown for a serum sample from a healthy control; commercial BPIFB1 antibody; an APS1 patient with ILD and BPIFB1 autoantibodies; and a non-APS1 ILD patient with BPIFB1 autoantibodies. Scale bar = 150μM (B) A competition assay to confirm the specificity of the bronchiolar staining pattern for BPIFB1 was performed by pre-incubating serum samples with an equivalent amount of commercial recombinant BPIFB1 protein or bovine serum albumin (BSA) and tested in the BPIFB1 RLBA. Representative results from 2 independent experiments. (C) A sequential immunoprecipitation was performed by taking radiolabeled BPIFB1 protein and immunoprecipitating twice with serum from subjects with (lanes 6–10) and without (lanes 1–3) BPIFB1 autoantibodies or a commercial BPIFB1 antibody (lane 4). A final immunoprecipitation was performed using a commercial BPIFB1 antibody and the remaining protein was run out on an agarose gel that was exposed to film. The signal intensities of the bands were quantified using Kodak Molecular Imaging Software and graphed as shown. At far left are 2 columns indicating the mean ± SD of the BPIFB1 positive and BPIFB1 negative subjects. *$P=0.036$.
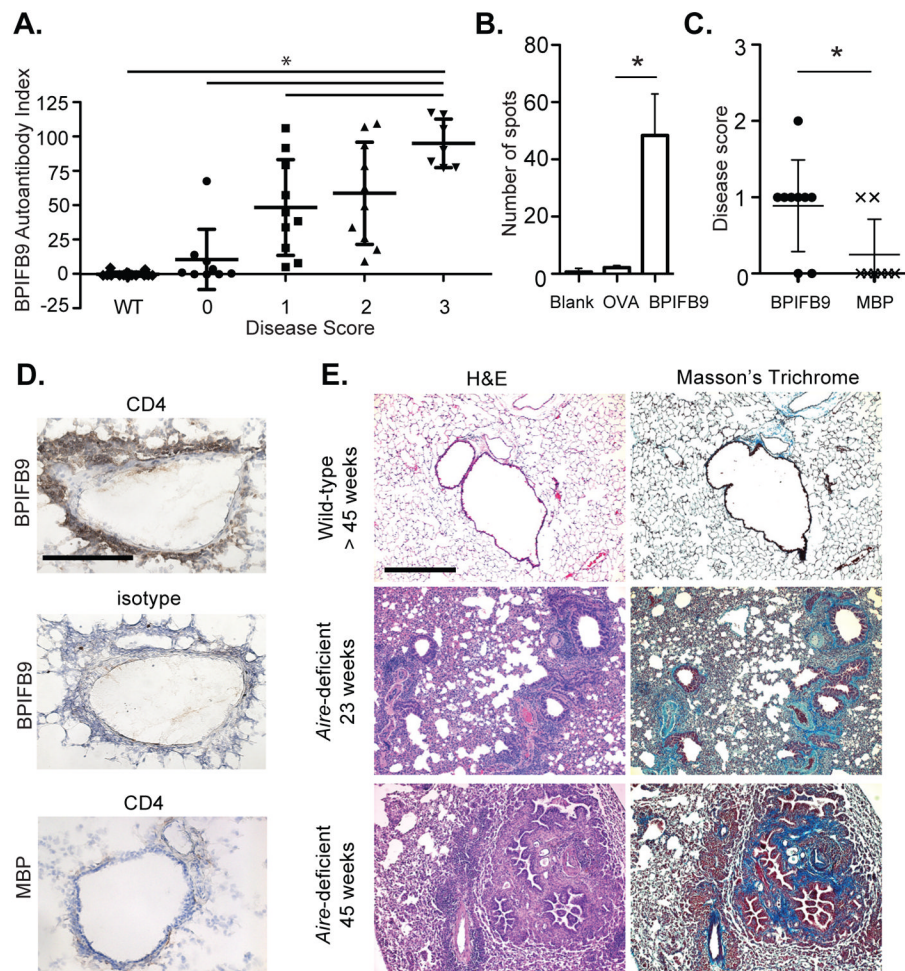
**Fig. 4. Autoimmunity to BPIFB9 induces interstitial pneumonia and pulmonary fibrosis in *Aire*<sup>−/−</sup> mice**

(A) *Aire*$^{-/-}$ (n=36) and wild-type mice (n=19) were analyzed for BPIFB9 autoantibody titer by RLBA and histologic lung disease score (0–3). Shown is the score of individual mice and the mean score ± SD for each group. The bars indicate statistically significant differences *P=0.0001 (WT vs. 3), P=0.0002 (0 vs. 3), P=0.0185 (1 vs. 3).* (B) Elispot analysis for IFNγ-secreting cells specific for BPIFB9 in immunized *Aire*$^{-/-}$ mice. Shown are the cumulative results of 2 independent experiments indicating the mean number of spots+SD for number of spots counted in each group, run in triplicate. *P=0.005.* (C) Lung disease score of recipient mice after adoptive transfer of BPIFB9-specific (n=9) or MBP-specific (n=8) CD4$^+$ T cells. *P=0.035* (D) Immunohistochemistry stain of CD4 T cells in lungs harvested from recipient mice following adoptive transfer of BPIFB9-specific or MBP-specific CD4$^+$ T cells (E) Serial sections from aged mice sacrificed at the indicated time points and analyzed for lung disease by H&E and Masson's trichrome stains. Scale bar = 200μM.
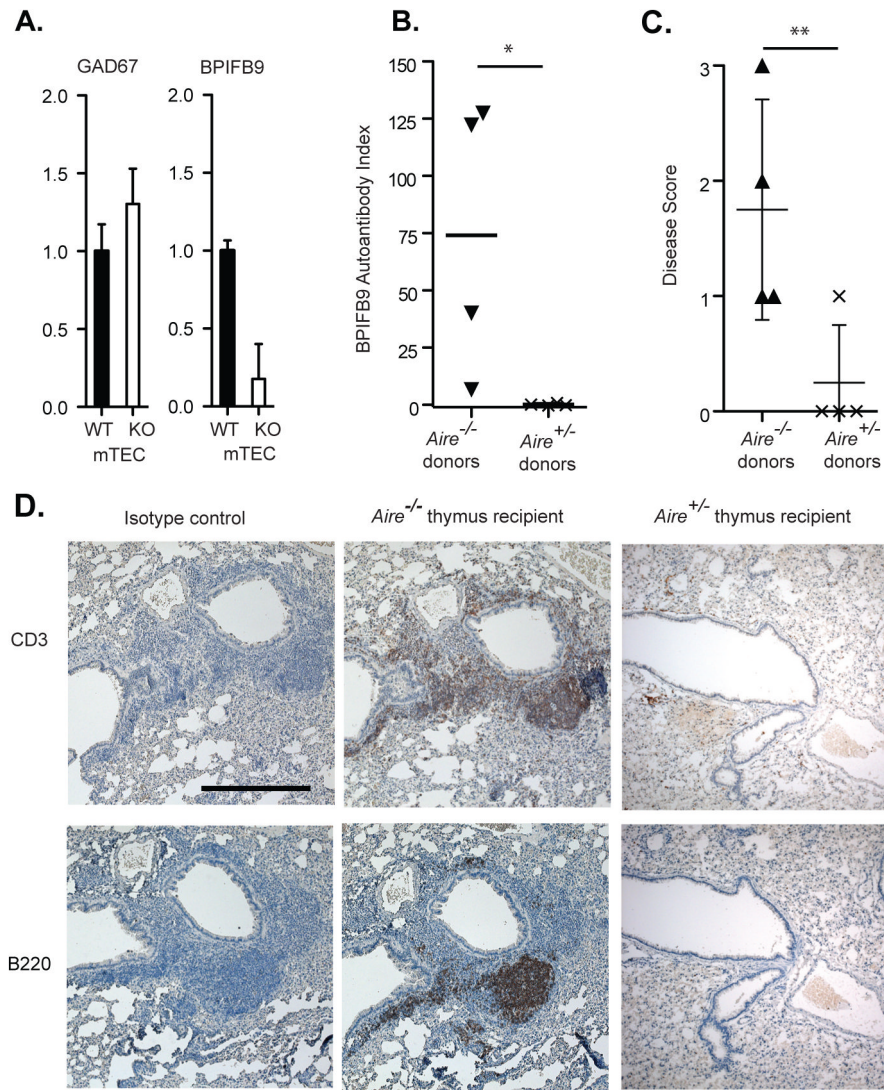
**Fig. 5. Loss of BPIFB9 expression in *Aire*⁻/⁻ thymi leads to BPIFB9 autoantibodies and lung disease after thymic transplantation**
(**A**) Expression of BPIFB9 mRNA in mTECs from *Aire*+/+ and *Aire*−/− mice (n=5 for each). Glutamatic acid decarboxylase-67 (GAD67) is an Aire-independent tissue specific antigen control. Results representative of 3 independent experiments. (**B**) RLBA results from recipient mice 10 weeks post-thymic transplant of *Aire*−/− and *Aire*+/− grafts (n=4 for each). *P=0.029. (**C**) Lung disease score of the thymic transplant recipients. **P=0.055. (**D**) Representative immunostaining of lungs from thymic transplant recipients for T cells (CD3) and B cells (B220).
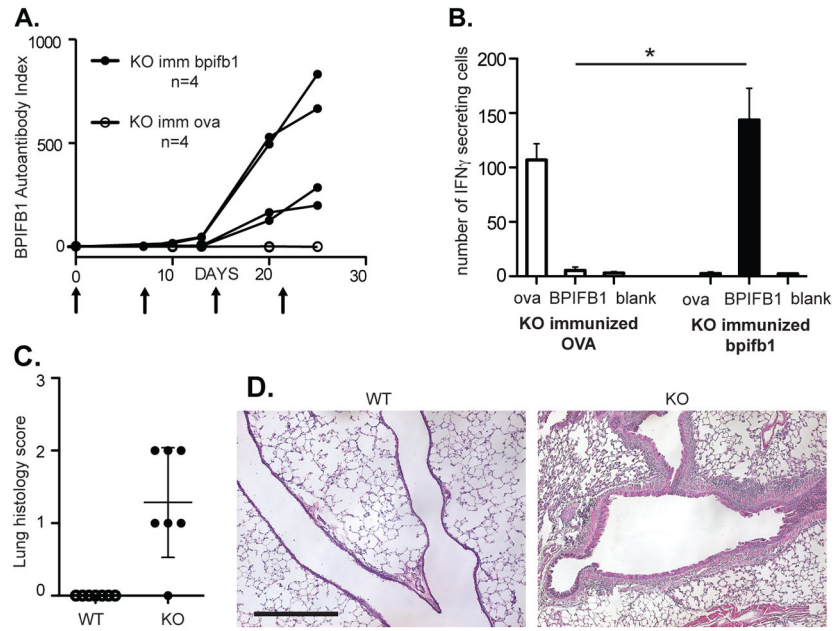
**Fig. 6. Adoptive transfer of BPIFB1-specific B and T cells leads to ILD**

(A) Radioligand binding assay to quantify BPIFB1-specific autoantibodies in *Bpifb1*$^{-/-}$ mice immunized with ovalbumin or BPIFB1 protein. The arrow indicates day immunized. (B) Elispot analysis for IFNγ-secreting cells specific for BPIFB1 or ovalbumin harvested from lymph nodes of immunized *Bpifb1*$^{-/-}$ mice shown in A. Cumulative results from 2 independent experiments. *P=0.005. (C) Lung histology scores of *Rag2*$^{-/-}$ mice after adoptive transfer of lymphocytes harvested from wild-type (n=7) or *Bpifb1*$^{-/-}$ (n=7) mice after immunization with BPIFB1 protein. Cumulative results from 2 independent experiments shown. (D) Representative H&E sections of lungs from mice shown in C.

**Table 1**

*AIRE* genotype and clinical data for APS1 patients with elevated BPIFB1 autoantibodies.

| APS1 patient | *AIRE* gene mutation | APS1 criteria | Clinical Data | Clinical course | BPFIB1 antibody status |
|---|---|---|---|---|---|
| 1 | 62C>T/ 1096-1G>A | AD[1], MCC[2], HP[3] | **PFT**: obstruction<br>**Imaging** : scattered ground glass opacities, centrilobular nodules, bronchiectasis<br>**Biopsy**: diffuse lymphocytic bronchiolitis | Respiratory exacerbations with wheezing, improvement with steroids and mycophenolate | + |
| 2 | 607C>T/769C>T | AD, MCC, HP | **PFT**: obstruction<br>**Imaging**: bronchiectasis | Antibiotic treatment for recurrent pulmonary infections, respiratory failure, death | + |
| 3 | 769C>T/769C>T | AD, MCC | **PFT**: restriction, obstruction<br>**Imaging**: organizing pneumonia, bronchiectasis<br>**Biopsy**: bronchiolitis obliterans, lymphoid aggregates | Wheezing, steroid treatment, respiratory failure, death | + |
| 4 | 964del13/964del13 | AD, HP, MCC | **PFT**: restriction, obstruction<br>**Imaging**: centrilobular nodules, bronchial wall thickening, bronchiectasis, ground glass opacities<br>**Biopsy**: peribronchiolar inflammation | Chronic cough, antibiotic treatment for recurrent pulmonary infections, respiratory failure, death | + |
| 5 | 964del13/964del13 | AD, HP, MCC | **PFT**: restriction, impaired diffusion<br>**Imaging**: diffuse interstitial and ground glass opacities, centrilobular nodules<br>**Biopsy**: lymphocytic bronchiolitis, interstitial inflammation | Cough, dyspnea, improvement with immunosuppression | + |
| 6 | 967_979del13/967_979del13 | HP, MCC | **PFT**: obstruction<br>**Imaging**: centrilobular nodules, bronchiectasis<br>**Biopsy**: lymphocytic bronchiolitis, non-specific interstitial pneumonia, lymphoid aggregates | Respiratory failure, intubation, improvement with immunosuppression | + |
| 7 | 769C>T/769C>T/ | AD, HP | N/A[4] | N/A | + |
| 8 | 769C>T/769C>T | AD, MCC | N/A | N/A | + |
| 9 | 769C>T/769C>T | AD, HP | N/A | N/A | + |
| 10 | 769C>T/769C>T | AD, HP, MCC | N/A | N/A | + |

[1] Addison's disease;

[2] mucocutaneous candidiasis;

[3] hypoparathyroidism;

[4] not available.