Pulmonary autoimmunity as a feature of autoimmune polyendocrine syndrome type 1 and identification of KCNRG as a bronchial autoantigen

Mohammad Alimohammadi[a,1], Noémie Dubois[b,c], Filip Sköldberg[a], Åsa Hallgren[a], Isabelle Tardivel[b], Håkan Hedstrand[a], Jan Haavik[d], Eystein S. Husebye[e,f], Jan Gustafsson[g], Fredrik Rorsman[a], Antonella Meloni[h], Christer Janson[a], Bernard Vialettes[c], Merja Kajosaari[i], William Egner[j], Ravishankar Sargur[j], Fredrik Pontén[k], Zahir Amoura[l], Alain Grimfeld[m], Filippo De Luca[n], Corrado Betterle[o], Jaakko Perheentupa[i], Olle Kämpe[a], and Jean-Claude Carel[b,p]

Departments of [a]Medical Sciences and [g]Women's and Children's Health, University Hospital, and [k]Department of Genetics and Pathology, the Rudbeck Laboratory, Uppsala University, 751 85 Uppsala, Sweden; [b]Institut National de la Santé et de la Recherche Médicale, Unité 561, Groupe Hospitalier Cochin–Saint Vincent de Paul, F-75674 Paris, France; [c]Departments of Nutrition, Metabolic Diseases, and Endocrinology, Timone Hospital, Université de la Méditerranée, 13005 Marseille, France; [d]Department of Biomedicine, University of Bergen, 5009 Bergen, Norway; [e]Division of Endocrinology, Institute of Medicine, University of Bergen, 5021 Bergen, Norway; [f]Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway; [h]Pediatric Division II, 'Microcitemico' Hospital, Department of Biomedical and Biotechnological Sciences, University of Cagliari, 09124 Cagliari, Italy; [i]The Hospital for Children and Adolescents, University of Helsinki, Box 281, Fin-00029 HUS, Helsinki, Finland; [j]Sheffield Teaching Hospitals National Health Service Foundation Trust, Clinical Immunology and Allergy Unit, Northern General Hospital, Sheffield S5 7AU, United Kingdom; [l]Service de Médecine Interne, Hôpital Pitié-Salpêtrière and Université Pierre et Marie Curie Paris 6, 75013 Paris, France; [m]Department of Pediatric Pulmonology and Allergy, Armand Trousseau Hospital, University Pierre and Marie Curie, 75012 Paris, France; [n]Department of Pediatrics, University of Messina, 98126 Messina–Gazzi, Italy; [o]Department of Medical and Surgical Sciences, University of Padua, 35128 Padua, Italy; and [p]Department of Pediatric Endocrinology and Diabetology, Robert Debré Hospital and University Paris 7 Denis Diderot, Institut National de la Santé et de la Recherche Médicale, Unité 690, 75019 Paris, France

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Patients with autoimmune polyendocrine syndrome type 1 (APS-1) suffer from multiple organ-specific autoimmunity with autoantibodies against target tissue-specific autoantigens. Endocrine and nonendocrine organs such as skin, hair follicles, and liver are targeted by the immune system. Despite sporadic observations of pulmonary symptoms among APS-1 patients, an autoimmune mechanism for pulmonary involvement has not been elucidated. We report here on a subset of APS-1 patients with respiratory symptoms. Eight patients with pulmonary involvement were identified. Severe airway obstruction was found in 4 patients, leading to death in 2. Immunoscreening of a cDNA library using serum samples from a patient with APS-1 and obstructive respiratory symptoms identified a putative potassium channel regulator (KCNRG) as a pulmonary autoantigen. Reactivity to recombinant KCNRG was assessed in 110 APS-1 patients by using immunoprecipitation. Autoantibodies to KCNRG were present in 7 of the 8 patients with respiratory symptoms, but in only 1 of 102 APS-1 patients without respiratory symptoms. Expression of KCNRG messenger RNA and protein was found to be predominantly restricted to the epithelial cells of terminal bronchioles. Autoantibodies to KCNRG, a protein mainly expressed in bronchial epithelium, are strongly associated with pulmonary involvement in APS-1. These findings may facilitate the recognition, diagnosis, characterization, and understanding of the pulmonary manifestations of APS-1.

Autoimmune polyendocrine syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy [APECED, Online Mendelian Inheritance in Man (OMIM) 240300], is a rare disorder caused by mutations in the autoimmune regulator (*AIRE*) gene (1). Patients with APS-1 progressively develop multiple organ-specific autoimmunity of endocrine and nonendocrine tissues. Loss of function of the Aire protein results in decreased expression of self-antigens in medullary thymic epithelial cells and in failure to establish central tolerance to a range of different autoantigens (2). Multiple autoantibodies directed against specific intracellular autoantigens are found. Well-defined autoantigens in APS-1 include 21-hydroxylase in the adrenal cortex, tryptophan hydroxylase in intestinal serotonin-producing cells, and NACHT, leucine-rich repeat, and PYD containing 5 (NALP5) in parathyroid glands (3–5). Detection of autoantibodies can help in the diagnosis of a disease component or predict its future development (6). Hypoparathyroidism and Addison's disease are the most frequent disease components, and >20 different autoimmune manifestations have been identified in APS-1 (1, 7, 8). MHC and non-MHC allelic variation are believed to influence disease expression (9, 10). Identification of tissue-specific autoantigens in APS-1 provides useful diagnostic markers and increases our understanding of the variable expression of disease in individuals (6).

Although pulmonary disease has been sporadically observed in APS-1 patients, an immune-mediated mechanism has not been established (8, 11). We describe APS-1 patients with autoimmune pulmonary disease and a potassium channel-regulating protein preferentially expressed in the epithelial cells of terminal bronchioles (KCNRG) as the putative target antigen.

Results

Identification of Airway Inflammation as a Component of APS-1. Severe respiratory symptoms were seen in 4 of 110 APS-1 patients described below (patients 1–4, Table 1) Five additional cases with respiratory involvement and/or KCNRG autoantibodies were subsequently identified after recruitment to the study (patients 5–9, Table 1).

Patient 1 developed asthma-like respiratory symptoms at 5 years of age. At age 10, the symptoms worsened and were poorly controlled by inhaled and oral glucocorticoids. By 11, APS-1 was diagnosed. Lung function tests showed obstruction with reduced forced vital capacity (FVC), forced expiratory volume (FEV_1), and forced expiratory flow (FEF 25–75%) at 73%, 48%, and 26% of predicted. Skin prick tests were negative for all allergens tested. Chest CT scan showed bronchiectasis with peribronchial

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[1]To whom correspondence should be addressed. E-mail: mohammad.alimohammadi@medsci.uu.se.

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Table 1. Description of the APS-1 patients with respiratory symptoms or KCNRG antibodies

Patient no. (country of origin)	Age of onset of pulmonary manifestations	Initial pulmonary symptoms	Pulmonary function tests/X-rays/pulmonary histology	Management	Outcome	Antibodies to KCNRG	APS-1 manifestations	*Aire* gene mutation
1 (France)	Childhood (≈5 y of age)	Obstructive symptoms with frequent exacerbations	Obstructive pulmonary disease/ground glass opacities, bronchiectasis/12 y: bronchiolopathy and peribronchiolar inflammatory infiltrate	Steroids, azathioprine, mycophenolate mofetil	Steroid dependence: marked improvement on mycophenolate mofetil	+	Candidiasis, hypoparathyriodism, Addison's disease, alopecia, pernicious anemia, exocrine pancreas insufficiency	62C > T/ 1096-1G > A
2 (Italy)	Childhood	Several lower respiratory tract infections from 5 y of age	Obstructive pulmonary disease/10 y: bilateral bronchiectasis	Antibiotics, supportive care	Death at 18 y due to cor pulmonale and terminal respiratory failure.	+	Candidiasis, hypoparathyroidism, Addison's disease, pernicious anemia, intestinal dysfunction/malabsorbtion	607C > T/ 769C > T
3 (Finland)	Childhood	Asthma-like symptoms	Obstructive and restrictive pulmonary disease/bronchiectasis/ 10 y: bronchiolopathy, bronchiolitis obliterans	Steroids	Severe respiratory failure	+	Candidiasis, hepatitis, intestinal dysfunction/malabsorption, Addison's disease	769C > T/ 769C > T
4 (France)	Childhood	Chronic cough	Obstructive then restrictive pulmonary disease/20 y: bronchiectasis/34 y: peribronchiolar inflammatory infiltrate	Antibiotics, supportive care	Exacerbations with recurrent pulmonary infections; death at 37 y of chronic respiratory failure	+	Candidiasis, hypoparathyroidism, Addison's disease, hepatitis	964del13/ 964del13
5 (United Kingdom)	Childhood (11 y)	Cough, dyspnea, thoracic pain	Restrictive pulmonary disease with decreased DLCO/bilateral interstitial infiltrate and peribronchial ground-glass opacity/lymphocytic bronchiolitis	Hydroxychloroquine from 11–18 y	Improvement at 20 y	-	Candidiasis, hypoparathyroidism, Addison's disease, hepatitis, ectodermal dysplasia, specific antibody deficiency (failure to respond to polysaccharide vaccine and recurrent bacterial infections), on i.v. immunoglobulin	964del13/ 964del13
6 (Sweden)	Young adulthood	Airway hyperresponsiveness and obstructive symptoms	N.D.	Inhaled terbutaline and budesonide, acetylcysteine	Mild to moderate symptoms of bronchial hyperresponsiveness.	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, alopecia, vitiligo	769C > T/ 769C > T
7 (Sweden)	Young adulthood	Airway hyperresponsiveness	N.D.	Inhaled terbutaline, acetylcysteine	Improvement	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, hepatitis, intestinal dysfunction/malabsorption	769C > T/ 769C > T
8 (Sweden)	Young adulthood	Airway hyperresponsiveness; respiratory symptoms; infection-induced exacerbations	N.D.	Inhaled terbutaline, acetylcysteine	Improvement; occasionally, mild respiratory symptoms	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, intestinal dysfunction/malabsorption, hepatitis, gastritis	M388fsX36/ C62 > T
9 (Norway)	N.A.	No respiratory symptoms	N.D.	N.A.	No respiratory symptoms	+	Candidiais, hypoparathyroidism, Addison's disease, hypogonadism, intestinal dysfunction/malabsorption	967-979del13/ 967-979del13

N.D., not done; N.A., not applicable. Seven of 8 patients with respiratory symptoms had antibodies to KCNRG, whereas KCNRG antibodies were positive in only 1 APS-1 patient without respiratory symptoms.

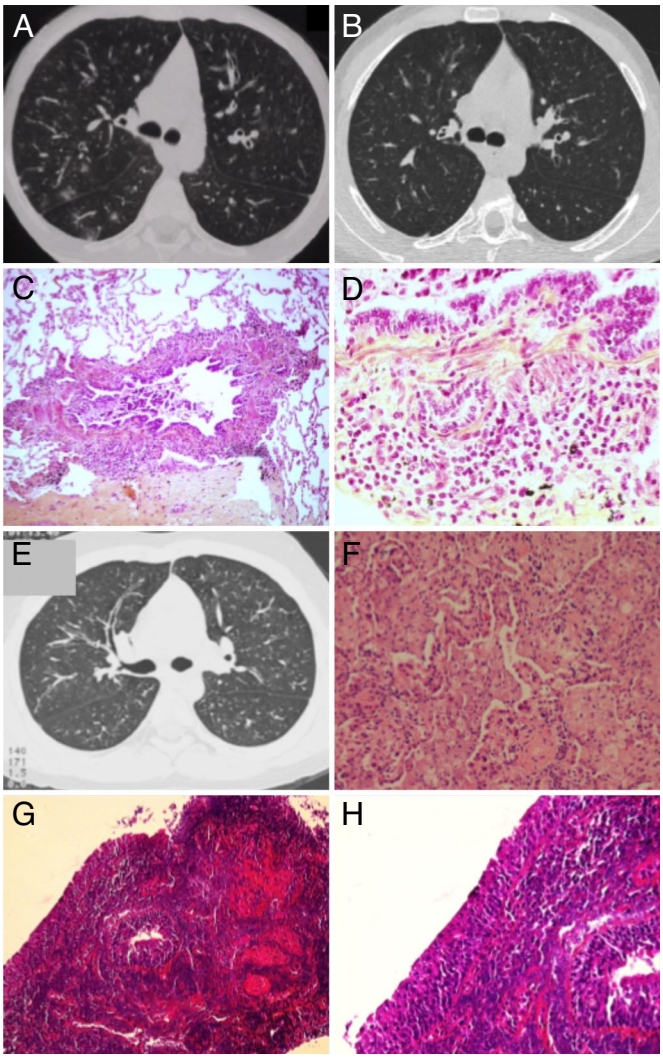

Fig. 1. Radiological and histological aspects of pulmonary involvement in APS-1 patients. (*A*) Pulmonary CT scan in patient 1 at the age of 11 years: peribronchial ground glass opacities. (*B*) Pulmonary CT scan in patient 1 at the age of 16 years, on immunosuppressive treatment with mycophenloate mofetil; the peribronchiolar abnormalities are improved. (*C* and *D*) Histological appearance of lung biopsy in patient 1 at the age of 11 years [10× magnification (*C*) and 40× magnification (*D*)]: peribronchiolar lymphoid infiltrate. (*E*) Pulmonary CT scan in patient 3 showing established bronchiectasis. (*F*) Lung biopsy on patient 3 showing bronchiolitis obliterans organizing pneumonia. (*G* and *H*) Histological appearance of lung biopsy in patient 4, at the age of 35 years, 2 years before his death from chronic respiratory failure [10× magnification (*G*) and 40× magnification (*H*)] showing severe peribronchiolar infiltrate.

ground-glass opacities (Fig. 1*A*). Treatment with prednisolone (2 mg/kg/d) markedly improved the respiratory symptoms, but relapses were severe upon dose reduction. Lung biopsy showed inflammation with peribronchiolar lymphoid infiltrate in the small bronchioles (Fig. 1 *C* and *D*). Azathioprine was started at 14 years of age with little steroid-sparing effect. Mycophenolate mofetil (750 mg twice daily) was commenced at 15.5 years of age with good effect, and prednisolone was decreased to 0.14 mg/kg/d. Respiratory symptoms and CT scan appearances improved, and improvement was maintained at evaluation 2.5 years later (Fig. 1*B*).

Patient 2 was diagnosed with APS-1 in early childhood but died at the age of 18 of cor pulmonale and respiratory failure. An autopsy was not performed. From the age of 5, he had lower respiratory tract infections at least 2–3 times a year. Chest X-ray and CT scan revealed bilateral bronchiectasis from 10 years of age. Lung function tests showed reduced FEV_1 (<40%) and FVC at 50–60% of predicted. His respiratory symptoms deteriorated with exercise intolerance, shortness of breath, and growth failure. Exacerbations were poorly controlled by cycles of antibiotic and glucocorticoid therapy. Chronic colonization with *Burkholderia cepacia* developed. By 14 years of age, he was oxygen dependent with FEV_1 and FVC at 14% and 13%, respectively, of expected. Other causes were excluded by extensive investigations including sweat test, nasal mucosal brush biopsy, and genetic analysis for cystic fibrosis.

Patient 3, currently 19 years old, was diagnosed with hepatitis due to APS-1 at 9 months of age. Dyspnoea in early childhood was initially diagnosed as asthma. By 10 years of age, bronchiolitis obliterans organizing pneumonia had developed, with bronchiectasis on the CT scan verified by lung biopsy (Fig. 1 *E* and *F*). He suffered recurrent lower respiratory tract infections. He is currently oxygen dependent, with an FVC and FEV_1 at 31% and 18%, respectively, of predicted.

Patient 4 presented with a chronic cough in childhood. APS-1 was diagnosed at 9 years of age. By age 20, he had established bronchiectasis on chest X-ray and CT scan. Spirometry showed airway obstruction. The patient had frequent episodes of "infectious bronchitis" and gradually deteriorated. At 34 years of age, he was admitted to intensive care because of hypoxemic pneumonia. Lung biopsy showed a severe peribronchiolar infiltrate (Fig. 1 *G* and *H*). He died of chronic respiratory failure at the age of 37.

Immunoscreening of a cDNA Library and Autoantibody Assay on KCNRG. By immunoscreening a bovine cDNA library with serum from patient 6 with obstructive pulmonary disease and hypoparathyroidism, we found 3 independent clones encoding KCNRG (GenBank accession no. AY190923). KCNRG-specific autoantibodies were subsequently sought in sera from 105 APS-1 unselected patients independent of the presence of respiratory symptoms. Four of these 105 sera (patients 6–9 in Table 1, including patient 6, used for immunoscreening) were positive. None of 252 control sera were positive (Fig. 2*A*). These findings led us to test for immunoreactivity to KCNRG in sera from APS-1 patients selected for the presence of severe pulmonary disease (patients 1–5, Table 1). Four of these 5 patients (patients 1–4, Table 1) had high-titer antibody. In total, 8 of 110 (7.2%) APS-1 patients investigated displayed the KCNRG autoantibodies.

Expression Analysis of KCNRG Messenger RNA and Protein. Microarray expression databases, such as GNF SymAtlas and GeneNote, state that tissue expression of KCNRG is almost ubiquitous (12, 13). Nevertheless, we investigated the tissue expression of KCNRG by Northern blot analysis and quantitative real-time PCR. Northern blot analysis [supporting information (SI) Fig. S1] demonstrated that expression of KCNRG was actually restricted to the lungs. Quantitative PCR analysis (Fig. 2*B*) showed that mRNA expression of KCNRG was predominantly restricted to the lungs. However, it also revealed that KCNRG mRNA was expressed to a low extent in the pancreas and prostate. Nevertheless, expression in other tissues was at much lower levels than in lungs. Immunohistochemistry on bovine lung using a rabbit polyclonal antiserum developed against KCNRG specifically stained epithelial cells of the terminal bronchioles (Fig. 3*B*). To further exclude the ubiquitous expression of the KCNRG at protein level, we used the antiserum directed against KCNRG and stained a human multitissue array. The results were in line with the results for mRNA expression experiments (Fig. S2).

Immunostaining of Lung Tissue with APS-1 Sera. Immunofluorescence of bovine lung with anti-KCNRG-positive sera from

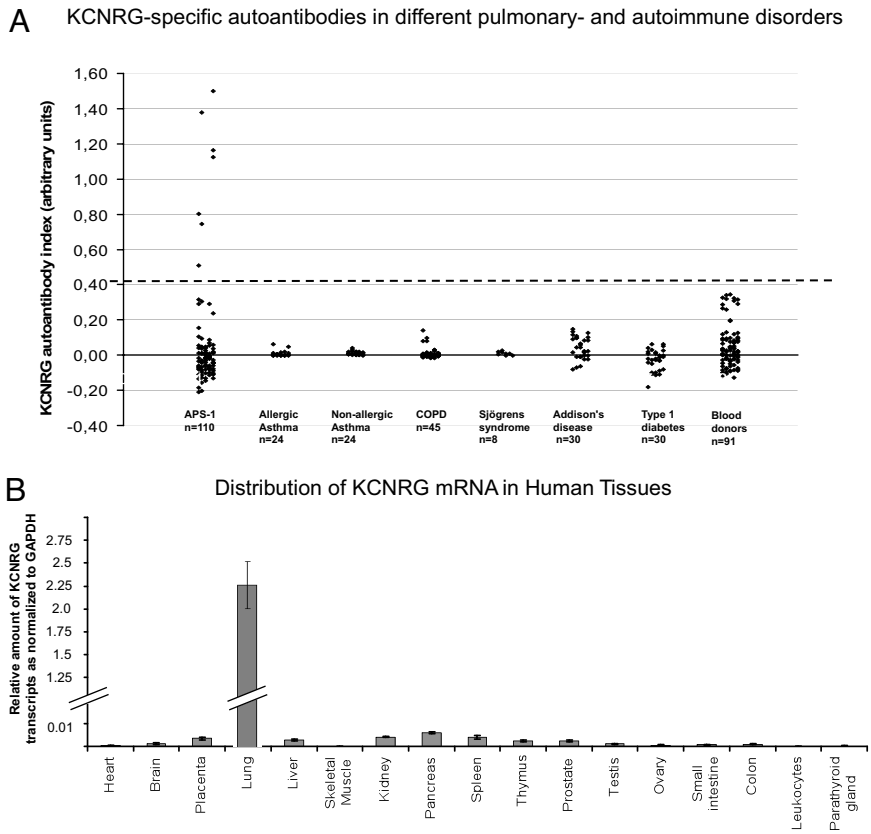

Fig. 2. Autoantibody reactivity to KCNRG and tissue expression of KCNRG messenger RNA. (*A*) Comparison of KCNRG autoantibody titers in sera from APS-1 patients, patients with different pulmonary disorders or other autoimmune disorders, and healthy blood donors. The assay for detection of autoantibodies is described in *Materials and Methods*. The dashed line indicates a cut of value of 0.41, which is the upper level of normal, defined as the mean results obtained for the healthy blood donors + 3 SD. (*B*) Expression of KCNRG mRNA in adult human tissues as measured by quantitative PCR, demonstrating that the expression of KCNRG is mainly restricted to pulmonary tissue. Please note the noncontinuous *y* axis.

APS-1 patients showed specific staining of the epithelial cells of terminal bronchioles with 2 of the 3 sera used (Fig. 3 *E* and *F*). The staining pattern of patient sera was identical to that of the KCNRG antiserum. No staining was seen with control sera from anti-KCNRG-negative APS-1 patients (Fig. 3 *H* and *I*) or healthy blood donors (Fig. 3 *J* and *K*).

The specificity of APS-1 patient autoantibodies for KCNRG was confirmed by preabsorption studies. In these experiments, preabsorption of the patient serum samples with recombinant KCNRG abolished the staining of the terminal bronchioles (Fig. S3 *B* and *E*). In contrast, preabsorption of the sera with an equal amount of Luciferase did not reduce specific staining of the terminal bronchioles (Fig. S3 *C* and *F*).

Discussion

We present evidence showing that pulmonary autoimmunity is a component of APS-1 and have identified the KCNRG protein as a target autoantigen. Strong serum reactivity against KCNRG was found in most APS-1 patients (7 of 8) with respiratory involvement of varying severity—with fatal outcomes in some cases. We demonstrate that KCNRG expression is mainly restricted to the epithelial cells of terminal bronchioles. These findings have significance for clinicians who care for patients with APS-1 and provide a tool to define and investigate the possible pulmonary autoimmunity in APS-1 and thereby distinguish it from concurrent obstructive lung disease or lower respiratory tract infections. The terminal bronchiole is a previously unrecognized autoimmune target in APS-1.

Symptoms displayed were quite variable (Table 1). Patients 6–8 had relatively mild symptoms, well controlled by inhaled β_2-agonists and inhaled glucocorticoid and mucolytic drugs, that may go unnoticed in the context of APS-1 where several disabling disease components can mask less-obvious symptoms. In contrast, 4 patients (patients 1–4, Table 1) had severe respiratory symptoms, initially asthma-like but evolving into severe obstructive lung disease with radiological signs of bronchiectasis. Two died from pulmonary disease at 18 and 37 years of age, and another patient is oxygen dependent at the age of 18, but patient 1 displayed dramatic improvement when treated with mycophenolate mofetil immunosuppression. In patients 1, 2, and 4, the relationship between the respiratory symptoms and APS-1 was not considered initially, even when cystic fibrosis had been ruled out. All of these 4 patients had high-titer autoantibodies to KCNRG, and early knowledge of this may have influenced their management. KCNRG autoantibodies are, however, discordant with pulmonary manifestations in 2 APS-1 patients (patients 5 and 9). Patient 5 was negative for KCNRG autoantibodies when her initial respiratory symptoms appeared. A presumed lymphocytic interstitial pneumonia (LIP) had remitted after hydroxychloroquine treatment. However, in addition to APS-1, this patient had recurrent pulmonary bacterial infections due to antibody deficiency. She responded well to i.v. Ig replacement therapy (IVIG) but has had recurrent unexplained respiratory symptoms thought to be due to LIP. This treatment may have altered this patient's pulmonary presentation and/or masked the detection of autoantibodies. To our knowledge, this patient is the only case of APS-1 on IVIG therapy. Patient 9 was positive for

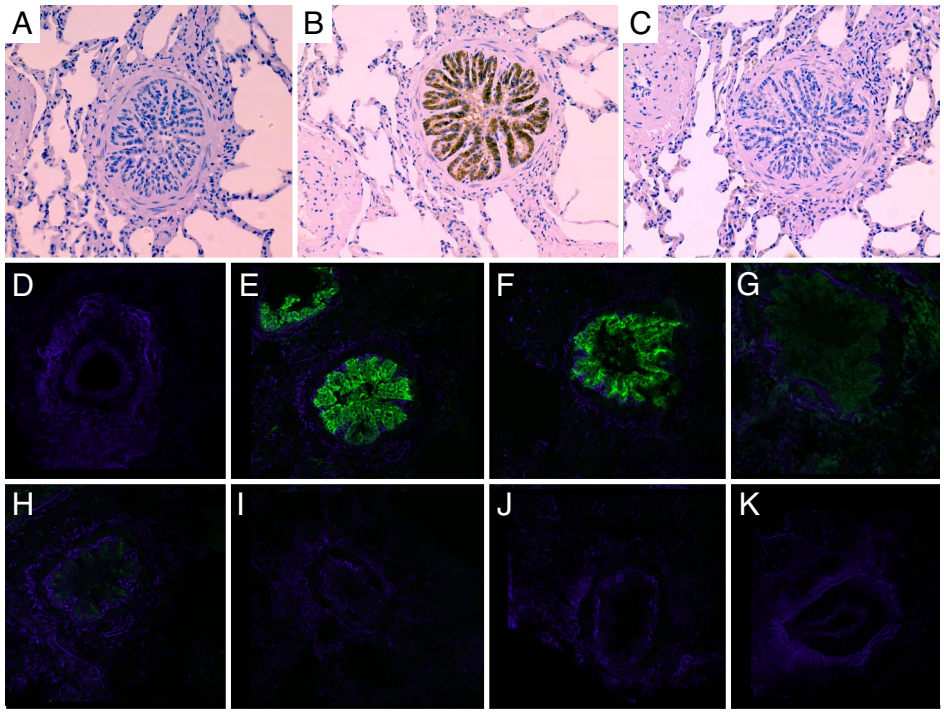

Fig. 3. Distribution of KCNRG protein in bovine lung. (*A–C*). Immunohistochemistry on 4-μm paraffin-embedded sections, using affinity-purified anti-KCNRG rabbit antiserum. (*A*) Background staining without the primary antibody. (*B*) Antiserum used in dilution 1:1000. (*C*) Antiserum in dilution 1:1,000 preabsorbed with 20 nmol of the peptide used for the immunization. (*D–K*) Immunofluorescence on 6-μm cryosections of lung, using patient and control serum samples. The blue background is from DAPI that stains nucleoli. FITC-conjugated goat anti-human IgG secondary antibody (green) was used. (*D*) Background staining when no primary serum is used. (*E–G*) Staining with 3 APS-1 patients' sera with KCNRG reactivity. (*H* and *I*) Staining with 2 APS-1 sera without KCNRG reactivity. (*J* and *K*) Staining with sera from 2 healthy blood donors.

KCNRG autoantibodies but has no respiratory symptoms or pathological signs on chest X-ray, spirometry, or plethysmography. In addition, it should be taken into consideration that respiratory disease in this report was defined from patients' self-reported symptoms and not by prospective lung-function tests. It may therefore be possible that some additional cases of patients with undetected respiratory disease may exist in our cohort. These possible cases apparently do not show autoantibody response to KCNRG.

The absence of a perfect correlation between presence of KCRNG autoantibodies and respiratory manifestation is not surprising. It parallels other autoantibody responses in APS-1 and other autoimmune conditions where only a fraction of autoantibody-positive individuals manifest the clinical disease component at any time point (14, 15). There may also be heterogeneity of the immune response in APS-1, and other pulmonary autoantigens could be targeted. Because we could only test a single serum sample from each patient, it is impossible to rule out fluctuation of KCNRG autoantibodies over time. Alternatively, APS-1 patients may present with confounding respiratory symptoms due to common intercurrent diseases (asthma and infections) or *Candida* infections.

Although pulmonary autoimmunity hitherto has not been considered as a component of APS-1 in humans (8, 16), the animal model for APS-1, *Aire*-deficient mice display pulmonary pathology of variable severity depending on the background strain. *Aire*-deficient mice on C57BL6 and BALB/c background display modest pulmonary disease, whereas *Aire*-deficient mice on NOD and SJL background strain have severe and fatal lung pathology comparable with the histological appearances in our APS-1 patients (10). Phenotypic variability may therefore depend on genetic background in humans.

Is KCNRG a valid candidate bronchial autoantigen? Northern blot analysis and quantitative real-time PCR analysis demonstrate predominant expression in lungs, and immunohistochemistry localizes the KCNRG protein to epithelial cells of small bronchioles. Two human splice variants of KCNRG encoding 31- and 26-kDa isoforms have been characterized by us (Figs. S4 and S5) and others (17). KCNRG has a homology to the cytoplasmic tetramerization domain of voltage-gated potassium channels and KCNRG inhibits potassium fluxes in vitro, suggesting that KCNRG may function as a potassium channel-regulating protein (17). We have experimentally confirmed the tendency of KCNRG to form tetramers in vitro (Fig. S6). Although the exact role of KCNRG in the lung remains to be determined, a role of potassium channels in histamine-induced bronchoconstriction and plasma exudation has been postulated, and drugs interfering with potassium channels have been proposed to treat bronchoconstriction (18, 19). It is also well recognized that autoantibodies to calcium and potassium channels can cause autoimmune disease such as the Lambert–Eaton myasthenic syndrome (20).

We have identified KCNRG, a putative potassium channel-regulating protein expressed in bronchial epithelial cells, as an autoantigen in APS-1 associated with pulmonary manifestations. We report pulmonary autoimmunity as a disease component in APS-1 with a potentially fatal outcome. Early recognition of pulmonary autoimmunity, and its distinction from asthma and recurrent bacterial infections is important because the autoimmune bronchiolitis in APS-1 may respond well to immunosuppression. Our findings also highlight APS-1 as a condition that provides an important opportunity to study autoimmunity in the lungs.

Materials and Methods

Ethics Approval. Informed written consent was obtained for all participants. Ethics committee approval was obtained from the Uppsala University (Permit UPS-02-415).

Patients and Sera. Serum samples were analyzed from 110 APS-1 patients (11 Swedish, 18 Norwegian, 58 Finnish, 18 Italian, 4 French, and 1 from the United Kingdom) with at least 2 of the major clinical components of APS-1 (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 (102 of the 110 patients); all of the 9 patients with KCNRG autoantibodies had typical mutations in the *Aire* gene]; detection of respiratory symptoms (because of the high number of included patients from several centers in 6 different countries, we could not systematically perform lung function examination on the entire cohort; hence, respiratory symptoms described here were defined from patient self-report of dyspnoea or cough, leading to relevant pulmonary work-up to exclude other causes of respiratory symptoms). Detailed information on each of the patient's respiratory symptoms is included in *Results*. Control sera were obtained from patients with allergic asthma ($n = 24$), nonallergic asthma ($n = 24$), chronic obstructive pulmonary disease (COPD) ($n = 45$), Sjögren's syndrome with respiratory symptoms ($n = 8$), Addison's disease ($n = 30$), and type 1 diabetes ($n = 30$) and from healthy blood donors ($n = 91$) (see also Table S1).

Construction and Screening of cDNA Expression Library. Messenger RNA was isolated from bovine tissue, obtained at a local abattoir. A cDNA expression library was constructed in the λ-ZAP Express vector (Stratagene). The library was immunoscreened with serum from an APS-1 patient (patient 6, Table 1) as previously described (21). Isolated clones were sequenced, and their DNA and deduced amino acid sequences were analyzed by using the Basic Local Alignment Sequence Tool (BLAST) (22).

Generation of ^{35}S-Labeled Human KCNRG and Immunoprecipitation/RIA for KCNRG Autoantibodies. The KCNRG-encoding clone, isolated by immunoscreening of the cDNA library, was used as template for coupled in vitro transcription, translation, and labeling with [^{35}S]methionine by using the TnT system (Promega) (23). Autoantibody reactivity against the clones was determined by immunoprecipitation, followed by analysis of the immunoprecipitates on SDS/PAGE, and/or evaluation of the precipitated radioactivity on an automated β counter as previously described (24, 25).

Expression Analysis by Quantitative PCR and Northern Blot. Complementary DNA from normal human tissues obtained from BD Biosciences were normalized and used as templates for quantitative PCR analysis on an iCycler MyiQ (Bio-Rad). Primer sequences, PCR conditions, and conditions for the Northern blot analysis is provided in the *SI Text*.

KCNRG Antiserum Generation and Immunoblotting. An antiserum against KCNRG was raised by immunization of rabbits with the peptide LPPQRPSYHDLVFQC, present in both human and bovine KCNRG and affinity-purified on a peptide column. Specificity was confirmed by immunoblotting with bovine lung total protein extract and by absorption studies in which the reactivity was blocked by preincubation with the peptide used for immunization.

Immunohistochemistry. Samples of bovine lung were fixed and paraffin-embedded. Sections of 4 μm thickness were deparaffinized, microwave treated, blocked, and incubated overnight at 4 °C with the KCNRG antiserum (dilution 1:1,000). The slides were then washed, exposed for 30 min to a biotinylated secondary antibody, and developed by using the VECTASTAIN ABC system (Vector Laboratories) and ChemMate DAKO Envision Detection kit (DAKO). Negative control slides were used for comparison.

Immunofluorescence and Laser-Scanning Confocal Microscopic Analysis. Cryosections (6 μm) of bovine lung tissue were air-dried, blocked, and incubated with APS-1 patient sera with KCNRG reactivity (dilution 1:400). The slides were incubated with FITC conjugated secondary antibodies (dilution 1:200) for 30 min. Slides were analyzed on a Zeiss LSM 510 confocal microscope. Sera from healthy blood donors and from APS-1 patients without KCNRG-specific autoantibodies were used as negative controls.

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1. Ahonen P, Myllarniemi S, Sipila I, Perheentupa J (1990) Clinical variation of autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med* 322:1829–1836.
2. Anderson MS, et al. (2002) Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395–1401.
3. Alimohammadi M, et al. (2008) Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. *N Engl J Med* 358:1018–1028.
4. Ekwall O, et al. (1998) Identification of tryptophan hydroxylase as an intestinal autoantigen. *Lancet* 352:279–283.
5. Winqvist O, Karlsson FA, Kämpe O (1992) 21-Hydroxylase, a major autoantigen in idiopathic Addison's disease. *Lancet* 339:1559–1562.
6. Betterle C, Dal Pra C, Mantero F, Zanchetta R (2002) Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: Autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. *Endocr Rev* 23:327–364.
7. Neufeld M, Maclaren N, Blizzard R (1980) Autoimmune polyglandular syndromes. *Pediatr Ann* 9:154–162.
8. Perheentupa J (2006) Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. *J Clin Endocrinol Metab* 91:2843–2850.
9. Gylling M, et al. (2000) ss-cell autoantibodies, human leukocyte antigen II alleles, and type 1 diabetes in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. *J Clin Endocrinol Metab* 85:4434–4440.
10. Jiang W, Anderson MS, Bronson R, Mathis D, Benoist C (2005) Modifier loci condition autoimmunity provoked by Aire deficiency. *J Exp Med* 202:805–815.
11. De Luca F, et al. (2008) Sicilian family with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) and lethal lung disease in one of the affected brothers. *Eur J Pediatr* 167:1283–1288.
12. Shmueli O, et al. (2003) GeneNote: Whole genome expression profiles in normal human tissues. *C R Biol* 326:1067–1072.
13. Su A, et al. (2002) Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci USA* 99:4465–4470.
14. Ekwall O, Sjöberg K, Mirakian R, Rorsman F, Kämpe O (1999) Tryptophan hydroxylase autoantibodies and intestinal disease in autoimmune polyendocrine syndrome type 1. *Lancet* 354:568.
15. Hedstrand H, et al. (2001) The transcription factors SOX9 and SOX10 are vitiligo autoantigens in autoimmune polyendocrine syndrome type I. *J Biol Chem* 276:35390–35395.
16. Betterle C, Greggio NA, Volpato M (1998) Autoimmune polyglandular syndrome type 1. *J Clin Endocrinol Metab* 83:1049–1055.
17. Ivanov DV, et al. (2003) A new human gene KCNRG encoding potassium channel regulating protein is a cancer suppressor gene candidate located in 13q14.3. *FEBS Lett* 539:156–160.
18. Kidney JC, Lötvall JO, Lei Y, Chung KF, Barnes PJ (1996) The effect of inhaled K^+ channel openers on bronchoconstriction and airway microvascular leakage in anaesthetised guinea pigs. *Eur J Pharmacol* 296:81–87.
19. Small RC, Berry JL, Foster RW (1992) Potassium channel opening drugs and the airways. *Res Braz J Med Biol Res* 25:983–998.
20. Vincent A (2006) Immunology of disorders of neuromuscular transmission. *Acta Neurol Scand Suppl* 183:1–7.
21. Rorsman F, et al. (1995) Aromatic-L-amino-acid decarboxylase, a pyridoxal phosphate-dependent enzyme, is a beta-cell autoantigen. *Proc Natl Acad Sci USA* 92:8626–8629.
22. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
23. Husebye ES, et al. (1997) Autoantibodies against aromatic L-amino acid decarboxylase in autoimmune polyendocrine syndrome type I. *J Clin Endocrinol Metab* 82:147–150.
24. Falorni A, et al. (1995) High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant human 21-hydroxylase. *J Clin Endocrinol Metab* 80:2752–2755.
25. Falorni A, Örtqvist E, Persson B, Lernmark A (1995) Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies 35S or 3H recombinant human ligands. *J Immunol Methods* 186:89–99.