Materials and Methods

Mice

Aire$^{o/o}$ mice were generated as described *(1, 2)*. *Aire*$^{o/o}$ mice used in these experiments were backcrossed into the C57BL/6, BALB/c, and NOD ShiLt/J backgrounds >10 generations. BALB/c SCID mice were purchased from the Jackson Laboratory. All mice were housed in a pathogen-free barrier facility at UCSF. Experiments complied with the Animal Welfare Act and NIH guidelines for the ethical care and use of animals in biomedical research and were approved by the UCSF Animal Care and Use Committee.

Histology

Organs from mice were harvested and fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained for hematoxylin and eosin. Human specimens were stained by the UCSF Pathology Laboratory. Immune infiltrates of organs were confirmed by an independent reading of the slides with a blinded observer.

Lung histology scoring system

Lung histology sections were scored based on the following grading system: grade 0, normal lung; grade 1, infrequent perivascular and peribronchiolar mononuclear infiltrates; grade 2, frequent perivascular and peribronchiolar mononuclear infiltrates; grade 3, dense perivascular and peribronchiolar mononuclear infiltrates and interstitial pneumonia; grade 4, diffuse perivascular and peribronchiolar mononuclear infiltrates, interstitial pneumonia and architectural distortion.

Immunostaining

Immune cell subtypes were visualized by immunohistochemistry using antibodies

specific for CD4, CD8, and B220 (BD Biosciences) and a DAB staining kit (Vector Laboratories) on 7-micron frozen sections of *Aire*$^{o/o}$ mice cut on a cryostat. The human lung tissue biopsy was stained by the UCSF Pathology Laboratory.

Indirect immunofluorescence

Seven-micron frozen sections from SCID mice and a lung tissue sample provided by the UCSF Pathology Department taken from a patient who expired of non-pulmonary disease were fixed and blocked in PBS + 1% bovine serum albumin + 3% serum from same species as the secondary antibody, overnight at 4^{o}C. Primary incubation with mouse sera was at 1:100 for an hour and goat anti-mouse FITC (Jackson Immunoresearch) was used at 1:1000 for an hour. Primary incubation using human sera was at 1:100 for an hour and secondary incubation with donkey anti-human FITC (Jackson Immunoresearch) was at 1:2000 for an hour. An anti-LPLUNC1 antibody (Abnova, #H00092747-B01P) was used at 1:100 and incubated for an hour and goat anti-mouse FITC (Jackson Immunoresearch) was used at 1:1000 for an hour. Slides were examined on a microscope (Axiostar; Carl Zeiss MicroImaging, Inc.) with 5", 10", 20" and 40" lenses. Images were obtained using an AxioCam with AxioVision software (both from Carl Zeiss MicroImaging, Inc.).

Flow cytometry

Lungs were minced and then digested in 2 mg/ml collagenase Dulbecco's Modified Eagle Medium (DMEM) for 20 minutes. The remaining tissue was dispersed by vortexing and filtered through nylon mesh. Cells were placed in DMEM complete media with 10% FCS and Golgi-Stop (BD Biosciences) and stimulated with 10 ng/ml phorbol

12-myristate 13-acetate (PMA) and 0.5 μM ionomycin (Sigma-Aldrich) for 4 h at 37°C. After the incubation, cells were surface stained with antibodies specific for CD4, CD8, and CD45 to be able to sort lymphocytes, then permeabilized and stained with antibodies specific for IL-4, IL-10, IL-17A, IFN-γ, or isotype control (BD Biosciences). Cells were analyzed on a LSRII flow cytometer (BD Biosciences) *(3)*.

Immunoblotting

Sera were screened for the presence of autoantibodies by immunoblotting as described *(2)*. Sera from BALB/c, B6 and NOD *Aire*$^{o/o}$ and *Aire*$^{+/+}$ mice were used at a 1:600 dilution and incubated overnight. The secondary antibody was peroxidase-conjugated goat antibody to mouse IgG (Jackson immunoresearch) used at 1:20,000 on immunoblots (1:15,000 on multiscreen immunoblots) for 1 hr. For competition studies, sera were pre-incubated with serial dilutions of recombinant VM-MBP or MBP overnight at 4^{o}C. The anti-VM sera were used at 1:200 for 1hr, and a peroxidase-conjugated goat antibody to rabbit IgG secondary antibody was used at 1:10,000 for 1 hr. For the multi-organ blot protein loading control, a GAPDH antibody (Santa Cruz Biotech) was used at 1:1000, and a peroxidase-conjugated goat antibody to mouse IgG secondary (Jackson immunoresearch) antibody was used at 1:2000 for 1hr. Tissue lysates were prepared in 0.1% CHAPS buffer from frozen organs harvested from immunodeficient SCID mice.

Immunoaffinity purification

Immunoprecipitation of the VM autoantigen was performed with *Aire*$^{o/o}$ sera as described *(2)*. The autoantigen was isolated from BAL fluid obtained by lavaging with PBS the lungs of several perfused and bled SCID mice. Protein agarose A/G beads (1:1

ratio) were covalently coupled to *Aire*$^{o/o}$ serum that had known reactivity to the 80 kD protein and placed into a column. The columns were washed in 30 mL PBS, and BAL was passed through the matrix. Columns were washed with 30 mL PBS and washed again with 30 mL of 10 mM phosphate, pH 6.8. Column elutions were collected by passing 0.5 ml fractions of 100 mM glycine, pH 2.5, over the column and collecting the flow-through. Elutions from multiple runs were pooled and concentrated in a centrifugal protein concentrator (Sartorius). The isolated protein was run out on a pH 3-10, non-linear two-dimensional gel in MOPS buffer, coomassie stained and the excised spots were sent for peptide mass spectrometry analysis.

Preparation of cDNA from organs and RT-PCR

RNA from organs was prepared according to manufacturer's instructions with an RNA isolation kit (Stratagene). RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and poly-dT primers and the VM and cyclophilin cDNA was amplified for 35 cycles. The following VM primers were used: forward 5'-TCAGCTTGCT-GCCTGAAGGTCAA-3', reverse 5'-TCAGCTTGCTGCCTGAA-3'.

Real-time PCR

Real-time PCR was performed on cDNA prepared from DNase-treated RNA. Aire, insulin2, GAD67 and cyclophilin primers were used as described *(2)*. For VM, we used the ABI Taqman Gene Expression Assay kit (Applied Biosystems #Mm00723516_m1). Reactions were run on a sequence detection system machine (HT7900; Applied Biosystems). For analysis of target gene expression from thymic stromal cDNA, the standard curve method was used.

Fusion protein vectors

Recombinant proteins were produced by cloning full length cDNA sequences into the pMAL-C2X system. Lung cDNA was prepared as above. The VM primers used to generate the full length cDNA with restriction sites for subcloning were: forward 5'-CGAATTCTCAGCTTGCTGCCTGAAGGTCAA-3'; reverse 5'-ATGTCGACTCAGCTT-GCTGCCTGAA-3'. PCR products were subcloned into pMAL-C2X via restriction digestion, ligated and transformed into BL-21 bacteria for protein expression; protein was harvested according to the manufacturer's instructions (pMAL system, New England Biolabs).

Prediction and selection of VM peptides

We used an online computer program that relies on Average Relative Binding (ARB) matrices to predict peptide sequences that bind to MHC molecules *(4)*. For the parameters, we selected H-2 IA^d as the matrix platform, the predicted IC_{50} function as linear and the IC_{50} (nM) cut off value of 5000.

ELISPOT analysis

$CD4^+$ T cells from *Aire*$^{o/o}$ or *Aire*$^{+/+}$ mice BALB/c mice were isolated by cell sorting on a cytometer (MoFlo; Dako). The release of IFN-γ by CD4 T cells was measured by ELISPOT assay. In brief, plates (Immunospot M200; BD Biosciences) were coated with 2 μg/ml of anti–mouse IFN-γ mAb (BD Biosciences) and incubated overnight at 4°C. The plates were washed with PBS and blocked with DMEM containing 10% FCS for 2 h at 37 °C. The $CD4^+$ T cells and irradiated APCs from syngeneic mice were plated at a ratio of 1:4 and VM-MBP and MBP proteins added. The plates were incubated for 24 hrs at

37 ^{o}C in 10% CO_2 incubator. At the completion of the incubation, the plates were washed thoroughly with PBS before adding 2 μg/ml of biotin-labeled IFN-γ mAb (2 μg/ml; BD Biosciences) and incubating for 2 hrs at 4^{o}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). Positive spots in the plate membranes were examined using an ELISPOT reader system (Transtec; Cell Technology). The number of spot-forming cells was the average number of spots in duplicate wells.

Immunization

Six to 8 week old BALB/c *Aire*$^{+/+}$ mice were immunized subcutaneously on the back in 4 spots with 100μg of VM-MBP or MBP in an emulsion of 100μL CFA supplemented with mycobacterium tuberculosis H37RA (4mg/uL), using a modified EAE protocol *(5)*. Re-immunizations using 100μg of VM-MBP or MBP in 100μL IFA were given 1 and 4 weeks after first immunization. Mice were sacrificed 4 weeks after the last immunization and the organs analyzed for histology.

Adoptive transfer of VM specific T cells

BALB/c *Aire*$^{+/+}$ mice were immunized with 100μg VM-111 (NLEGMLADVLNTVES) or the chicken ovalbumin peptide 323-339 (ISQAVHAAHAEINEAGR) mixed in CFA in 4 different spots on the back. Ten days after immunization, lymph nodes and spleen were harvested. Cells were activated *in vitro* with 5μg/mL of peptide in DMEM complete containing 10% FCS. After 4 days, lymphocytes were harvested by density centrifugation gradient and 20x10^{6} cells in PBS were transferred into each 6–8 week old

BALB/c SCID mouse via tail vein injection.

Statistics

Data was analyzed with Prism software (GraphPad) using unpaired t-tests with a two-tailed 95% confidence interval and $P \leq 0.05$ considered significant.

Generation of ^{35}S-Radiolabeled VM, LPLUNC and KCNRG and autoantibody assay

We used full-length cDNA clones for VM (isolated as above), human LPLUNC1 (C20orf114, ATCC #MGC-14597) and KCNRG (ATCC #MGC-40406) for *in vitro* transcription and translation and labeling with ^{35}S-methionine using the TNT system kit (Promega). The ^{35}S-radiolabeled proteins immunoprecipitated with serum or positive control antibodies to LPLUNC1 (Abnova #H00092747-B01P, Sigma #HPA024256) were aliquoted in triplicate in 96-well PVDF filtration plates (Millipore). In each well, 20,000 counts per minute (cpm) of ^{35}S-proteins were used for immunoprecipitation. The radioactivity of the immunoprecipitated material was evaluated with the use of a liquid scintillation counter (Beckman Coulter). 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).

Human subjects:

All patients and controls were included in the study only after we had obtained informed written consent. The study protocol was reviewed and approved by the institutional review board at UCSF.

Proliferation measurements by thymidine incorporation

Lymphocytes were harvested from spleen and lymph nodes of peptide-immunized mice

(see above) and cultured with 5μg/mL of either VM-111 or OVA 323-339 peptide, for 4 days in humidified 37°C, 10% CO_2 incubator. Cultures were pulsed with 1.0μCi of [^{3}H] thymidine at 18 hours before termination of culture, harvested using an automated multiwall harvester and counted using a scintillation counter.

Glycosidase Treatment

BAL fluid from SCID lungs lavaged with PBS was treated with N-Glycosidase F according to the manufacturer's protocol (New England Biolabs, #P0704L)

Mass spectrometry (MS)

Protein samples excised from the gel were directly submitted to the Stanford University Protein and Nucleic Acid facility for mass mapping where the samples were subjected to tryptic digestion and mass analysis of the resulting peptides. Mass mapping was performed on an Applied Biosystems 4700 Proteomics Analyzer, a MALDI mass spectrometer that provides tandem (MS/MS) time-of-flight (TOF) optics to provide peptide structural information, in addition to high-accuracy MS data *(6)*.

Supplementary Figures and Legends

Figure S1

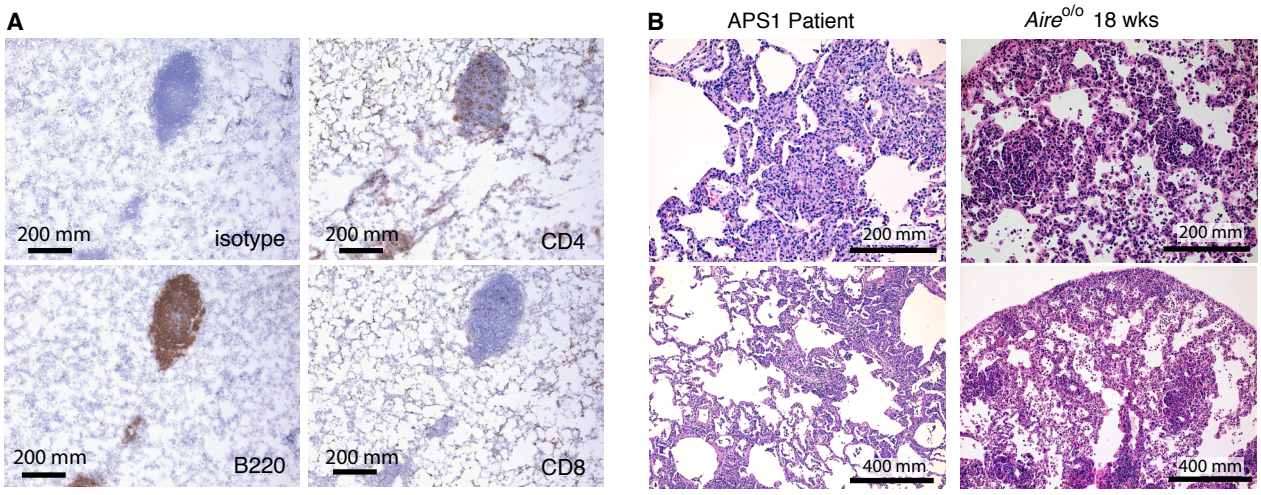

Fig. S1. Pulmonary disease in *Aire$^{o/o}$* mice and an APS1 patient. (A) Immunostaining of lungs from NOD *Aire$^{o/o}$* mouse at 8 weeks for CD4, CD8 and B cells. (B) Additional H&E stains demonstrating cellular interstitial pneumonia in an APS1 patient and a BALB/c *Aire$^{o/o}$* mouse at 18 weeks, both shown previously in Fig. 1A.

Figure S2

Patient Characteristics:	**Disease manifestations:**
Age: 3 years	- hypoparathyroidism
Sex: Female	- mucocutaneous candidiasis
	- dental enamel abnormalities
***AIRE* mutation:**	- vitiligo
homozygous for	- sicca syndrome
c.967_979del13	- recurrent fevers
	- constipation
	- respiratory disease

Fig. S2. Patient characteristics of an APS1 patient.

Figure S3

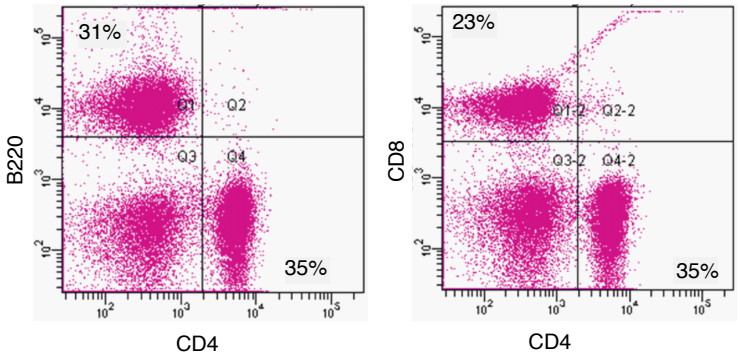

Fig. S3. Characterization of lung lymphocytes in *Aire$^{o/o}$* mice. Representative FACS plot gated on lung lymphocytes from a NOD *Aire$^{o/o}$* mouse at 10 weeks. Similar percentages were seen in both younger and older mice, as well as in BALB/c *Aire$^{o/o}$* mice.

Figure S4

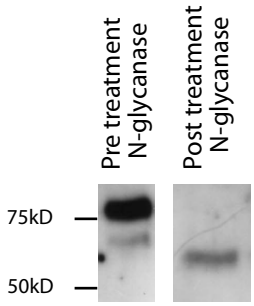

Fig. S4. Immunoblot of vomeromodulin after treatment with N-glycosidase. Immunoblot of BAL fluid probed with NOD *Aire*$^{o/o}$ mouse serum before and after treatment with N-glycosidase. The amount of post-treatment BAL analyzed was ~15% of the pre-treatment protein.

Figure S5

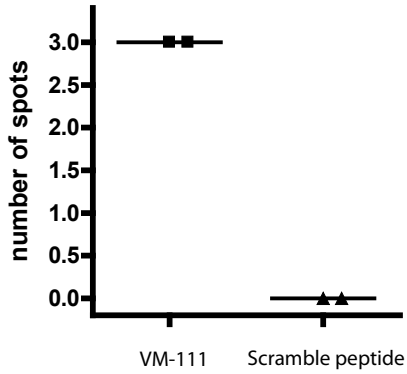

Fig. S5. ELISPOT of T cells in *Aire*$^{o/o}$ mice.
ELISPOT analysis of IFNg-producing T cells in two BALB/c *Aire*$^{o/o}$ mice 12 weeks of age immunized with full length VM. Y axis indicates number of spots per 90,000 CD4 T cells.

Figure S6

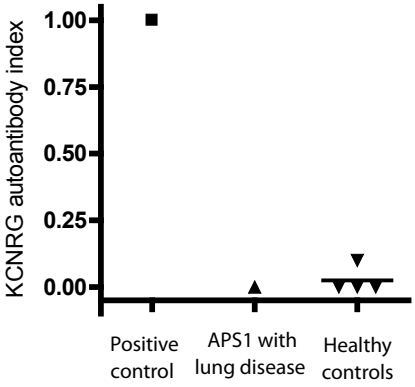

Fig. S6. KCNRG autoantibody assay in the APS1 patient with lung disease.

Supplementary References

1. M. S. Anderson, E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, et al, Projection of an immunological self shadow within the thymus by the aire protein. *Science.* **298,** 1395-1401 (2002).

2. J. DeVoss, Y. Hou, K. Johannes, W. Lu, G. I. Liou, J. Rinn, et al, Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J.Exp.Med.* **203,** 2727-2735 (2006).

3. J. J. Devoss, A. K. Shum, K. P. Johannes, W. Lu, A. K. Krawisz, P. Wang, et al, Effector mechanisms of the autoimmune syndrome in the murine model of autoimmune polyglandular syndrome type 1. *J.Immunol.* **181,** 4072-4079 (2008).

4. H. H. Bui, J. Sidney, B. Peters, M. Sathiamurthy, A. Sinichi, K. A. Purton, et al, Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications. *Immunogenetics.* **57,** 304-314 (2005).

5. I. M. Stromnes and J. M. Goverman, Active induction of experimental allergic encephalomyelitis. *Nat.Protoc.* **1,** 1810-1819 (2006).

6. J. T. Bechtel, R. C. Winant and D. Ganem, Host and viral proteins in the virion of Kaposi's sarcoma-associated herpesvirus. *J.Virol.* **79,** 4952-4964 (2005).