An Autoimmune Response to Odorant Binding Protein 1a Is Associated with Dry Eye in the Aire-Deficient Mouse

Jason J. DeVoss, Norbert P. LeClair, Yafei Hou, Navdeep K. Grewal, Kellsey P. Johannes, Wen Lu, Ting Yang, Craig Meagher, Lawrence Fong, Erich C. Strauss and Mark S. Anderson

*J Immunol* 2010; 184:4236-4246; Prepublished online 17 March 2010; doi: 10.4049/jimmunol.0902434

http://www.jimmunol.org/content/184/8/4236

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/03/15/jimmunol.0902434.DC1

References
This article cites 42 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/184/8/4236.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
An Autoimmune Response to Odorant Binding Protein 1a Is Associated with Dry Eye in the Aire-Deficient Mouse

Jason J. DeVoss,* Norbert P. LeClair,† Yafei Hou,‡ Navdeep K. Grewal,* Kellsey P. Johannes,* Wen Lu,* Ting Yang,* Craig Meagher,† Lawrence Fong,‡ Erich C. Strauss,† and Mark S. Anderson*,‡

Sjögren’s Syndrome (SS) is a human autoimmune disease characterized by immune-mediated destruction of the lacrimal and salivary glands. In this study, we show that the Aire-deficient mouse represents a new tool to investigate autoimmune dacryoadenitis and keratoconjunctivitis sicca, features of SS. Previous work in the Aire-deficient mouse suggested a role for α-fodrin, a ubiquitous Ag, in the disease process. Using an unbiased biochemical approach, however, we have identified a novel lacrimal gland autoantigen, odorant binding protein 1a, targeted by the autoimmune response. This novel autoantigen is expressed in the thymus in an Aire-dependent manner. The results from our study suggest that defects in central tolerance may contribute to SS and provide a new and clinically relevant model to investigate the pathogenic mechanisms in lacrimal gland autoimmunity and associated ocular surface sequelae. The Journal of Immunology, 2010, 184: 4236–4246.

Primary Sjögren’s Syndrome (SS) is an autoimmune disease characterized by a lymphocytic infiltrate in, and subsequent destruction of, the lacrimal and salivary glands (1). Immune-mediated impairment of glandular function results in keratoconjunctivitis sicca (KCS) and xerostomia (dryness of the eyes and mouth, respectively). However, these symptoms represent late-stage events and little is known about the pathologic mechanisms that result in a failure of immune tolerance.

Traditionally, animal models have been used to dissect the cellular and genetic mechanisms underlying many human diseases. A major barrier in understanding autoimmune disease, however, is the lack of knowledge of the inciting autoantigens. Multiple animal models of SS exist, including the NOD mouse, MRL/lpr, and more recently the C57BL/6.NOD-Aec1Aec2 (reviewed in Refs. 2–4); however, few autoantigens have been identified that are implicated in the disease process. The α-fodrin was identified as a potential autoantigen in mouse models (5–7) and in some human patients (8), although the relevance of this putative autoantigen to disease remains controversial (9). Anti–α-fodrin autoantibodies have been demonstrated in systemic lupus erythematosus, multiple sclerosis, Moyamoya, and glaucoma patients (10–13), suggesting that reactivity to α-fodrin may represent a generalized feature of autoimmunity or disease. Thus, the identification of additional autoantigens remains a priority for understanding SS.

In this study, we investigated autoimmune dacryoadenitis and associated dry eye complications in Aire-deficient mice, a mouse model of the human disease autoimmune polyglandular syndrome type 1 (APS1). The AIRE gene was identified as the defective gene in APS1 by positional cloning (14, 15). APS1 is an autosomal recessive, monogenic autoimmune disorder that results in immune-mediated destruction of a number of organs, predominantly of endocrine origin (16). Importantly, SS has also been described in a subset of patients with APS1 (16). In addition, KCS, a clinical hallmark of SS, has been described in a recent study of APS1 patients (17).

The animal model for APS1 has been critical in unraveling the cellular and molecular mechanisms of central tolerance that result in autoimmunity. Like their human counterparts, Aire-deficient mice develop spontaneous autoimmunity against multiple organs, including the retina, the salivary and lacrimal glands, the exocrine pancreas, the lungs, the liver, the stomach, and the reproductive organs (18). In this study, we describe a spontaneous dacryoadenitis that consumes the lacrimal tissue and results in defects in tear production, culminating in KCS, and damage to the ocular surface. Using an unbiased biochemical approach, we identified a novel autoantigen, odorant binding protein 1a (OBP1a), as a target of the immune response. This ∼18 kDa secreted protein is present in the lacrimal gland and in the tear fluid. The identification of this novel autoantigen and characterization of the spontaneous dacryoadenitis in Aire-deficient mice should be a useful new tool to study SS and allow the opportunity for more critical analysis of the pathogenesis of autoimmune-mediated ocular complications.

Materials and Methods

Mice

Aire-deficient mice were generated as previously described (18). Aire-deficient mice used in this study were backcrossed into the BALB/c and NOD Lt/J backgrounds greater than 10 generations. NOD.scid mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c nude...
mice were purchased from Taconic Farms (Germantown, NY). All mice were housed in a pathogen-free barrier facility at the University of California-San Francisco. Experiments complied with the Animal Welfare Act and National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the University of California-San Francisco Animal Care and Use Committee.

Ocular surface studies

Animals were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine prior to the procedure. The ocular surface was illuminated and imaged with a Nikon SMZ3000 stereozoom microscope and Nikon D100 digital camera. Lissamine green (Leiter’s Pharmacy, San Jose, CA) was applied to the ocular surface of mice at the 6-wk time point.

Histology and scoring

Organs from mice were harvested and fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained for H&E. Tissues were scored on a 4-point scale where: 0, no histological infiltrate; 1–4, 25% of the lacrimal gland is infiltrated; 2–26, 50% of the lacrimal gland is infiltrated; 3–51, 75% of the lacrimal gland is infiltrated, few ducts are present and fibrotic tissue is present; and 4–76, 100% of the lacrimal gland is infiltrated and no ducts are present.

Tear fluid collection

The 6- to 8-wk-old Aire-deficient and Aire-sufficient animals were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine prior to the procedure. To procure tear fluid, anesthetized animals were treated with 4.5 mg/kg pilocarpine. Tear fluid was collected using a Zone-Quik thread (FCI Ophthalmics, Marshfield Hills, MA) to the intercanthus of the eye for 15 s.

Immunoblotting

Sera were screened for the presence of autoantibodies by Western blotting as previously described (19). Briefly, lacrimal glands from 6-wk-old NOD, scid mice were homogenized on ice in Laemmli sample buffer (2% SDS, 10% glycerol, 5% 2-ME, 60 mM Tris-Cl, and 0.01% bromophenol blue). For tear fluid, 6-wk-old NOD,scid mice were anesthetized as described previously and stimulated with 4.5 mg/kg pilocarpine. Tear fluid was collected using a P10 pipette tip applied to the intercanthus of the eye and mixed with an equal volume of Laemmli sample buffer. Samples were incubated at 95 C for 10 min, then centrifuged at 13,000 rpm for 10 min. The extracts were separated by 10% Bis-Tris gel in MES buffer (In-vitrogen, Carlsbad, CA) and transferred to polyvinylidene fluoride membrane. The membranes were blocked with TBST (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk overnight, assembled in a Multiscreen apparatus (Bio-Rad Laboratories, Hercules, CA), and incubated with a 1:500 dilution of sera for 1 h. After washing five times with TBST, the bound Abs were reacted with HRP-conjugated donkey anti-mouse IgG (1:12,000; Bio-Rad Laboratories) for 1 h and revealed with an ECL reagent (Thermo Scientific, Waltham, MA) and autoradiography.

Immunofluorescence purification

Immunofluorescence purification of autoantigens was performed using protein agarose G-coupled to Aire-deficient or Aire-sufficient sera as described previously (20). Briefly, tissue extracts were prepared from immunodeficient mouse eyes (to reduce endogenous Ig contaminants) were homogenized in 0.15 M NaCl, 0.05 M Tris pH 8.0, and 0.1% CHAPS (Sigma-Aldrich). Protein agarose G-coupled columns were washed in 30 ml PBS, then immunodeficient eye extracts prepared in CHAPS buffer were passed through the matrix. Columns were washed with 30 ml PBS, then washed again with 30 ml 10 mM phosphate pH 6.8. Eluates were collected by passing 0.5 ml 100 mM glycine pH 2.5 over the column and collecting the flow through. Eluates from multiple runs were pooled and concentrated in a Vivaspin centrifugal protein concentrator (Sartorius, Aubagne, France).

In-gel digestion and peptide mass fingerprinting

OBP1a was identified by provisional peptide mass fingerprint (PMF) as previously described (21–24). Briefly, gel bands were excised, destained (stain-stripped) three times in 50% acetonitrile and 25 mM ammonium bicarbonate, pH 8.0, dehydrated with 100% acetonitrile, and dried in a Speed-Vac. Gel pieces were rehydrated with a solution of sequencing grade trypsin (10 µg trypsin [Promega, Madison, WI] in 25 mM ammonium bicarbonate) and the digestion was carried out for 16 h at 37 °C. Peptides were extracted three times by the addition of two volumes of an aqueous solution of 50% acetonitrile and 5% trifluoroacetic acid. The extracts were combined and reduced to a final volume of 5–10 µl. PMF was used for preliminary protein identification. Portions (typically 5%) of the unseparated tryptic digest was co-crystallized in a matrix of α-cyano-4- hydroxycinnamic acid (5 mg/ml) and analyzed on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) operating in reflector mode. Mass spectra were produced representing protonated molecular ions of tryptic peptides from the protein(s) present in each gel spot. The mass spectra were internally mass calibrated using two tryptic autolysis products present in the digest mixture (842.5100 Da and 2211.1046 Da). The mass measurement accuracy for all peptides was ±25 ppm, and the mass measurement precision, defined as the SD of differences between the experimental and theoretical peptide masses, was typically ±25 ppm. Preliminary protein identities were established by matching the experimentally determined peptide masses to those produced by an in silico tryptic digestion of the NCBI nr.2005.01.06 database within the window of experimental mass measurement accuracy. The PMF data-searching algorithm MS-Fit (http://prospector.ucsf.edu/cgi-bin/msform.cgi?form=msfitstandard) was used to perform the database searches.

Preparation of cDNA from organs

RNA from various organs was prepared according to manufacturers instructions using a mini RNA isolation kit (Stratagene, La Jolla, CA). RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and poly-dt primers.

Amplification of OBP1a and cyclophilin from organs

PCR was used to amplify OBP1a or cyclophilin from various organs. Primers used for OBPla: forward 5'-AGGCCAAAATTTCTGCTGC-3' and reverse 5'-TCATTCAAGGACGTAATCTG-3'. Cyclophilin primers were purchased from SuperArray Biosciences (Frederick, MD).

Fusion protein vectors

Recombinant proteins were produced by cloning full length cDNA sequences into pGEX-3X (GE Healthcare, Piscataway, NJ) or pMAL-C2X (New England Biolabs, Ipswich, MA). Briefly, RNA was prepared from lacrimal tissue using a RNA spin column (Stratagene) and converted into cDNA using Superscript III (Invitrogen) and oligo-dt primers. Primers specific for OBPla were used to generate ampiclon containing the full length sequence with restriction sites for cloning. For pGEX-3X, primers used were: forward primer 5'-CGGATCCGGCAAGCTATGAAATATCTGC-3' and reverse primer 5'-ATAGGATCCCGTATATGCAATTAAT-3'. These primers incorporated a BamHI site on the 5'-end and an NsiI site on the 3'-end of the ampiclon for subcloning. For pMAL-C2X, primers used were: forward primer 5'-TAATGATCCGGCAAGCTATGAAATATCTGC-3' and reverse primer 5'-TACTGGATCCGTCGACCACTGGAATAAT-3'. These primers incorporated a BamHI site on the 5'-end and a PstI site on the 3'-end of the ampiclon for subcloning Constructs were transformed into BL-21 bacteria for protein production.

Fusion protein production

BL-21 bacteria containing the recombinant protein vectors were grown overnight at 37 °C in 3 ml Luria broth with antibiotics. The following day, this culture was used to seed a 1:1 culture without antibiotics. When the culture reached an OD of 0.6, 0.01 mM isopropyl-β-D-thiogalactoside was added and the culture was incubated at 30 °C for an additional 3 h.

GST fusion protein purification

For GST constructs, fusion protein was harvested using a GST renaturation kit (Cell Biosystems, San Diego, CA). Briefly, cultures of BL-21 bacteria transformed with OBPla-GST were pelleted and resuspended in 1x STE buffer. Cells were lysed by sonication, then incubated in detergent solubilization buffer for 1 h on ice. The resulting supernatant fraction was collected by centrifugation at 15,000 × g for 10 min, then incubated for 1 h with detergent neutralization solution.

Glutathion Sepharose 4B (GE Healthcare, Waukesha, WI) was incubated with the supernatant containing fusion protein for 2 h, washed three times with PBS with 1% Triton X-100, then eluted in Laemmli sample buffer (2% SDS, 0.1 M DTT, 10% glycerol, 60 mM Tris). Recombinant protein was precipitated using chloroform and methanol then resuspended in 0.5% CHAPS buffer.

Malloose fusion protein purification

For maltose binding protein (MBP) constructs, fusion protein was prepared by lysing the bacteria by sonication, followed by centrifugation at 8000 g for...
30 min. Supernatants were collected and used for protein purification. Fusion protein was bound to amyllose resin on a column support and washed with 20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA (column buffer). Fusion proteins were eluted in column buffer supplemented with 10 mM maltose.

Removal of LPS from fusion proteins
LPS was removed from the fusion protein samples using Detoxifying Pierce Endogen, Rockford, IL) according to the manufacturer’s instructions.

Immunostaining
Immune cell subsets were visualized by immunohistochemistry using Abs specific for CD4 (clone RM4-5), CD8 (clone 53-6.7), and IgD (clone 11-26 x 2.2a; BD Pharmingen), a donkey anti-rat secondary Ab conjugated to HRP (The Jackson Laboratory) and a DAB staining kit (Vector Laboratories, Burlingame, CA). Briefly, lacrimal glands from 6- to 8-wk-old NOD Aire-deficient or Aire-sufficient animals were embedded in OCT Tissue Tek freezing media (Sakura Finetek, Torrance, CA). Thick sections, 10 μm, were prepared from these tissues using a cryostat (Leica, Wetzar, Germany) and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed for 10 min in acetone at -20°C, washed in PBS for 5 min, then blocked with 0.1 M Tris, 0.05 M NaCl, 0.1% Triton X-100 (PBS), 0.3% Tween 20, 3% normal donkey serum for 1 h at room temperature. After blocking, slides were incubated in 3% hydrogen peroxide to inactivate endogenous peroxidases, then incubated with primary appropriate Ab diluted 1:50 in block for 30 min at room temperature, washed three times with PBS for 5 min each, and incubated with secondary Ab diluted 1:200 in block for 30 min at room temperature. After additional washes in PBS for 5 min each, slides were developed with substrate and counterstained with H&E.

Preparation and flow cytometry from lacrimal gland
Whole lacrimal glands were digested in 1 mg/ml collagenase P (Roche Biosciences, Palo Alto, CA) in DMEM for 30 min at 37°C. Digested tissues were passed through a 70 μm cell strainer. Lymphocytes were prepared by centrifugation in Lympholyte M (Cedarlane Laboratories, Burlington, NC), according to manufacturer’s instructions. Lymphocytes were stained with Abs specific for mouse CD45 (clone 30-F11; BD Biosciences, San Jose, CA), CD4 (clone RM4-4; BD Biosciences), CD8 (clone 53-6.7; BD Biosciences), and CD19 (clone 6D5; Southern Biotechnology Associates, Birmingham, AL) and analyzed on a FACS Calibur (Becton Dickinson, San Diego, CA).

Adoptive transfer
Cervical lymph node cells (LNCs) and splenocytes were harvested and CD4+ or CD8+ T cells or CD19+ B cells were depleted using rabbit complement. Briefly, cells were incubated with CD4 (clone GK1.5), CD8 (clone YTS-169), or CD19 (clone ID3; BD Biosciences), followed by rabbit complement (Sigma-Aldrich) for 1 h at 37°C. Cells were analyzed by flow cytometry to assess removal of the desired cell population. Cell populations (5 x 10^6 CD4+ depleted, CD8+ depleted, or B cell depleted) were injected i.v. into NOD.scid mice. Animals were aged 40 d post-transfer, then sacrificed, and analyzed as described.

ELISPOTs
The 25,000 CD4+ T cells from the cervical LNCs or lacrimal gland infiltrating cells of 10- to 15-wk-old BALB/c Aire-deficient or Aire-wildtype animals were isolated by flow cytometry using a FACS Aria (Becton Dickinson). Purities were greater than 95% for all samples. For lacrimal gland infiltrating cells, the lacrimal glands were prepared as described previously and purified by flow cytometry using a FACS Aria. The 250,000 APCs from Aire wild-type BALB/c mice were used as target cells and cocultured with 5 μg/ml OB-P1a- MBP or MBP as the control protein for 24 h at 37°C in a 4% CO2 incubator. The release of IFN-γ by CD4 T cells was measured by ELISPOT assay. Briefly, Bi-T, Immumospot M200 plates (Becton Dickinson) were coated with anti-mouse IFN-γ mAb (2 μg/ml, no. 551216, BD Pharmingen, San Diego, CA) and incubated overnight at 4°C. The plates were washed with PBS and blocked with medium containing 10% FCS for 2 h at 37°C. The effector CD4+ T cells and irradiated APC (3000 rad) and Ag were added to each well and incubated for 24 h in RPMI 1640 complete medium. The plates were then washed thoroughly with PBS before adding biotin-labeled IFN-γ mAb (2 μg/ml, no. 554410, BD Pharmingen) and covering overnight at 4°C. After further incubation with Avidin-HRP (1:100 dilutions, no. 551950, BD Pharmingen) for 1 h at room temperature, the plates were developed using BD Pharmingen’s AEC substrate solution. Positive spots displayed in the plate membranes were examined using the Transtec ELISPOT reader system (Cell Technology, Columbia, MD). The number of spot-forming cells was the average number of spots in duplicate wells.

Adoptive transfer of stimulated cells
Cervical LNCs were harvested from 6-wk-old NOD.Aire-deficient mice. Twenty million cells were placed in culture with 50 μg/ml either of OB-P1a-MBP fusion protein or MBP protein alone. After 4 d in culture, lymphocytes were purified by Ficoll centrifugation. 1.5 million cells were adoptively transferred into NOD.scid recipients (The Jackson Laboratory) via i.v. injection. The mice were sacrificed 4 wk after transfer and analyzed by histology as described.

Immunostaining of adoptive transfer sections
Paraffin embedded tissues were cut into 5 μm sections on a cryotome and placed on glass microscope slides. Sections were deparaffinized and rehydrated (xylene, 100% ethanol, 95% ethanol; two changes each for 5 min per change). Ag retrieval was performed in 10 mM sodium citrate buffer pH 6 at 95°C for 30 min. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 15 min. Slides were blocked with 0.1 M Tris, 0.05 M NaPhos (PBS), 0.3% Tween 20, and 3% normal donkey serum for 1 h at room temperature. After blocking, slides were washed with TBST (three times, 5 min each) and incubated with isotype control or polyclonal rabbit anti-human CD3 (A0452, DakoCytomation, Carpinteria, CA) diluted 1:500 in block for 1 h at room temperature. Slides were washed again in TBST and a mouse anti-rabbit IgG biotinylated secondary was added for 1 h at room temperature. Slides were washed again in TBST and then sections were incubated with Elite ABC reagent for 15 min (Vector Laboratories). Sections were washed a final time in TBST and the reaction was developed with DAB. Sections were H&E counterstained and mounted.

Thymic stroma preparation
Thymic epithelial cells were prepared according to previously established protocol (25). Briefly, thymi from 5-wk-old BALB/c Aire-deficient or Aire-sufficient mice were removed and trimmed of fat. Small cuts into the capsule were made, and the thymi were gently agitated in 50 ml RPMI 1640 for 30 min at 4°C. Thymic fragments were manually dispersed via pipetting, recovered by settling, and digested with 0.125% collagenase D (Roche) with 0.1% DNase (Promega) in RPMI 1640 at 37°C. This digestion was repeated for a total of two times, and supernatants were retained. All supernatant fractions were pooled, and cells were collected by centrifugation at 400g for 10 min. CD45+ cells were removed by negative selection using CD45 microbeads (Miltenyi Biotec, Auburn, CA) and an AutoMACS instrument (Miltenyi Biotec). RNA was prepared from the CD45- fraction.

Real-time PCR
Real-time PCR was carried out on cDNA prepared from DNase treated RNA. Glutamic acid decarboxylase 67 (GAD67), and cyclophilin primers were used as previously described (18, 26). For OB-P1a, the following primers were used: Forward-5'-GAATGCAAGGAAATGAAAGTCACAT-3'; Reverse-5'-ATCTTCTTGCAAATATCCAGTGAT-3'; Probe-5'-FAM-TCAATGA- AAATGGACAGTGCTCATTGACCA-3'-TAMRA. For Sp1, the following primers were used: Forward-5'-GCTTTGGTGTCTCCATATCATGCCT-3'; Reverse-5'-ATACCGAGTCCGAATTGATGTG-3'; Probe-5'-FAM- TGGCCAGACCGGGAGACAAAAC-3'-TAMRA. Reactions were run on an Applied Biosystems HT7900 Sequence Detection System machine. For analysis of target gene expression from organ derived cDNA, the standard curve method was used as previously described (20).

Thymic transplantation
Thymi were removed from 1–2 d old BALB/c Aire-deficient or Aire-sufficient 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 d 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 6- to 8-wk-old adult nude mice on the BALB/c background (Taconic Farms, Hudson, NY). Four weeks after transplantation, animals were harvested and analyzed for T cell reconstitution by FACS and immune infiltrates by histology.

Statistics
Data was analyzed using Prism 4.0 (GraphPad, San Diego, CA) and a Mann-Whitney nonparametric test.
Results

Dry eye and ocular surface changes in Aire-deficient mice mimic SS

Because a small proportion of patients with APS1 also have the clinical symptoms of SS (16), we assessed the consequences of autoimmune-mediated lacrimal gland destruction and dysfunction in Aire-deficient mice. Initial studies focused on evaluation of the ocular surface in Aire-deficient mice using established ophthalmic techniques. In contrast with Aire-sufficient mice on the NOD background (Fig. 1A), Aire-deficient NOD mice at 5 wk of age demonstrated clinical signs of aqueous-deficient KCS consistent with human SS, including significant irregularity of the corneal epithelium and filamentary keratopathy (Fig. 1B; arrowheads), signaling inflammation, and stress on the corneal surface. At 6 wk of age, Aire-deficient NOD mice showed marked pathologic changes on the ocular surface, including extensive and confluent corneal epithelial defects (Fig. 1B). Ocular surface changes were also observed in Aire-deficient mice on the BALB/c background; however, the onset, severity, and penetrance of the pathologic signs were diminished (data not shown). No obvious changes in the cornea were observed in Aire-deficient mice on the C57BL/6 background (data not shown), although recent published data suggest that desiccating stress in Aire-deficient C57BL/6 mice results in keratoconjunctivitis (27). To demonstrate a possible correlation of the ocular surface findings in Aire-deficient NOD mice with lacrimal gland dysfunction, next we analyzed tear fluid production. Tear fluid production of 6- to 8-wk-old NOD Aire-deficient and Aire-sufficient mice was stimulated using pilocarpine, and total volume was measured using Zone-Quik threads (Fig. 1C). Our results revealed that Aire-deficient NOD mice had markedly diminished (p = 0.0002) tear fluid production as compared with Aire-sufficient animals with many Aire-deficient mice producing only trace amounts of tear fluid.

Autoimmune-mediated destruction of the lacrimal gland characterized by autoantibodies and immune infiltrates

To confirm that the observed change in the ocular surface and decreased tear production in Aire-deficient mice results from destruction of the lacrimal gland, we performed histological analysis of the lacrimal gland in 6- to 8-wk-old Aire-deficient and Aire-sufficient animals on the NOD background (Fig. 2A). Mononuclear infiltrates were present in the lacrimal glands of Aire-deficient mice, often consuming the entire organ. In contrast, only sparse infiltrates were present in Aire-sufficient NOD mice consistent with published observations (28). Furthermore, similar infiltrates were also present in BALB/c Aire-deficient mice (Supplemental Fig. 1). In both strains, there was no gender-dependence and autoimmunity was observed equally in both male and female mice, consistent with clinical observations in APS1. The infiltrates observed in Aire-deficient mice appear to surround ductal tissue. To assess whether autoantibodies targeted the lacrimal gland, we performed indirect immunohistochemistry using sera from wild-type and Aire-deficient mice. Autoantibodies present in the sera of Aire-deficient mice recognize and react with proteins present in or around the ducts, as shown in Fig. 2B. Importantly, no autoreactivity was observed with wild-type sera reflecting the specificity of the Aire-deficient immune response. A previous study suggested that α-fodrin may be an autoantigen in this model system (7), although α-fodrin is not a thymically regulated Ag and its expression is not restricted to the lacrimal gland. To further characterize the autoantigen or autoantigens targeted by the autoantibody response, we performed immunoblots of lacrimal gland protein extracts with Aire-deficient or Aire-sufficient sera on the NOD (Fig. 2C) and BALB/c (Fig. 2D) backgrounds. Immunoblotting revealed an 18 kDa autoantigen present in the lacrimal gland extract, a pattern of reactivity not compatible with any described form of α-fodrin. Given that the indirect immunohistochemistry suggested that the protein was periductal, we hypothesized that it may be a secreted protein and performed similar immunoblots comparing lacrimal gland extract and tear fluid samples (Fig. 2E). Again, reactivity to an 18 kDa autoantigen was observed in sera from Aire-deficient animals. Animals sacrificed at various time points showed that autoantibodies were present at 4 wk of age in NOD Aire-deficient mice and their frequency increased with age (Table I). NOD Aire-sufficient mice as old as 1 y were screened and this autoantibody reactivity was not observed (data not shown). The 18 kDa Ag observed in NOD Aire-deficient mice was not dependent on the NOD background, as similar autoantibody reactivity and histological infiltrates are observed in Aire-deficient mice on the BALB/c background (Supplemental Fig. 1).

Identification of the 18 kDa autoantigen as OBP1a

Using an unbiased biochemical approach, we immunoaffinity purified an 18 kDa protein identified by PMF as OBP1a (Fig. 3A) from whole eye extracts. The original screen was undertaken to identify the retina-specific protein interphotoreceptor retinoid binding protein (IRBP); however, OBP1a was also identified.
OBP1a is a putative pheromone transporter that is part of the lipocalin family (Fig. 3B). It is a secreted protein and contains an N-terminal signal sequence. At the genetic level, it is encoded on the x chromosome of mice and is composed of 7 exons that result in a transcript of 757 bp. This transcript is translated into a 163 aa protein, part of which is presumably cleaved off (the signal sequence) during maturation and export. It has a predicted N-linked glycosylation site at position 104, which may explain the multiple bands observed by immunoblotting and identified by mass spectrometry as OBP1a (Fig. 3A). Interrogating the available databases determined that this protein was not expressed in the eye, a result that was confirmed by screening cDNA libraries prepared from various organs. Rather, the only organs from which detectable levels of transcript were identified were the lacrimal gland and the vomeronasal organ (Fig. 3C). We therefore reasoned that the NOD -deficient sera before immunoblotting, reactivity to this protein, whereas age-matched Aire-sufficient (both wild-type [wt] and heterozygous [het]) do not. E. Tear fluid and lacrimal gland extracts prepared from NOD.scid animals were immunoblotted using pooled sera from three 6- to 8-week-old NOD.Aire-deficient animals or three 10-week-old NOD.Aire-sufficient controls.

To confirm that OBP1a was a target of autoantibodies present in Aire-deficient mice, we constructed an OBP1a-GST fusion protein that was produced in Escherichia coli. This fusion protein, consisting of the 18 kDa OBP1a and a 25 kDa GST tag, migrated at ~43 kDa. Immunoblotting of recombinant OBP1a-GST fusion protein with sera from Aire-deficient mice confirmed the presence of autoantibodies against OBP1a in both the NOD background (Fig. 3D) and the BALB/c background (Fig. 3E). Not all NOD -deficient mice that responded to OBP1a from lacrimal gland extracts were reactive to the OBP1a-GST fusion protein, presumably reflecting Ab specificities to native folded protein or glycosylated moieties. When recombinant OBP1a-GST was incubated with Aire-deficient sera before immunoblotting, reactivity against the 18-kDa band in whole mouse lacrimal extract was reduced (Fig. 3F). In contrast, incubation with an equal concentration of purified GST tag alone failed to reduce immunoreactivity. Similarly, recombinant OBP1a-GST, but not GST tag alone, was able to reduce immunoreactivity against mouse tear fluid extract (data not shown). From these experiments, we conclude that OBP1a is the 18 kDa autoantigen present in the tear fluid and lacrimal glands and targeted by Aire-deficient mice.

Lacrimal infiltrate in Aire-deficient mice includes B and T cells

To determine the nature of the infiltrate present in the lacrimal glands of Aire-deficient mice, we performed immunohistochemistry with Abs specific for the T cell markers CD4 and CD8 and the B cell marker IgD. In control tissues from age- and gender-matched Aire-sufficient animals, few immunolabeled cells are present. In contrast, the lacrimal glands of NOD -deficient mice are infiltrated with CD4+ and CD8+ T cells, as well as IgD+ B cells (Fig. 4A). To determine the relative amount of each cell type present in the lesions, we performed flow cytometry with cell-specific markers. Lymphocytes were prepared from infiltrated lacrimal glands and interrogated for cell-surface expression of CD4, CD8, and CD19. The majority of the cells present in the

Table I. Frequency of autoantibodies against OBP1a and histology in NOD Aire-deficient mice at various ages

<table>
<thead>
<tr>
<th>Age at Sacrifice (wk)</th>
<th>Autoantibody Positive</th>
<th>Autoantibody Negative</th>
<th>Positive (%)</th>
<th>Infiltrates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–6</td>
<td>6</td>
<td>4</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>6–8</td>
<td>5</td>
<td>3</td>
<td>62.5</td>
<td>100</td>
</tr>
<tr>
<td>8–10</td>
<td>13</td>
<td>1</td>
<td>93</td>
<td>100</td>
</tr>
</tbody>
</table>
lesions, as suggested by immunohistochemistry, are CD45+CD19+ B cells. This finding was also confirmed in BALB/c Aire-deficient mice (Supplemental Fig. 2). The presence of a large proportion of B cells in the immune infiltrates, contradicting data as to the relevance of B cells in the Aire-deficient mouse model (29, 30), and the demonstration that autoantibodies are important in the pathogenesis of other mouse models of SS (31) led us to further investigate whether B cells were playing a role in lacrimal autoimmunity in the Aire-deficient mouse model. In an attempt to resolve whether one cell population was more important than the others in the disease process, we adoptively transferred lymphocytes depleted of individual cell populations into immunodeficient hosts. The purity of the transferred fractions was confirmed by FACS analysis prior to transfer (CD4 depleted: 1.2% CD4+, 8.7% CD8+, 57.7% CD19+; CD8 depleted fraction: 64.1% CD4+, 0.9% CD8+, 22.1% CD19+; B cell depleted: 57.4% CD4+, 30.4% CD8+, 3.7% CD19+). Consistent with previous experiments (20, 29), the transfer of CD4 depleted populations has a significantly lower average histological score, indicating an important role for CD4+ T cells in the disease process. In contrast, CD8 or B cell depleted lymphocytes were equally capable of mediating lacrimal disease in immunodeficient recipients (Fig. 5). Coupled with our previous data showing that lacrimal autoimmunity is not reduced in Aire-deficient, B cell-deficient animals (29), these data suggest that B cells play a limited role in lacrimal gland autoimmunity in this model.
OBP1a is expressed in the thymus in an Aire-dependent fashion

The current model of Aire function postulates that Aire upregulates self-Ag expression in the thymus. This model, however, was called into question with the identification of a-fodrin as a candidate autoantigen in lacrimal gland autoimmunity (7) and led many groups to postulate that Aire functions to shape T cell tolerance through mechanisms in addition to tissue-specific Ags (TSA) upregulation. To determine whether OBP1a was expressed in the thymus under the control of Aire, we purified thymic stroma from Aire-sufficient and Aire-deficient mice. cDNA generated from these cells was used to interrogate known Aire-regulated and Aire-independent TSAs, as well as OBP1a, using quantitative real-time PCR. OBP1a was expressed in the thymus in an Aire-regulated manner (Fig. 6). To confirm the accuracy of this analysis, SPT1, a known Aire-regulated Ag, was also shown to be Aire-dependent. In contrast, GAD67, which is known to be Aire-independent, was not Aire-regulated. Thus, the expression of OBP1a in the thymus appears to be Aire-dependent.

An Aire-deficient thymus is sufficient for disease

The transplantation of Aire-deficient stroma into Nude recipients was shown to result in retinal, stomach, and salivary autoimmunity (18, 32), supporting a critical role for the thymus in autoimmunity in Aire-deficient mice. To ascertain whether a defect in thymic TSA expression would result in lacrimal autoimmunity, we transplanted thymi from BALB/c Aire-sufficient and Aire-deficient newborn mice (1–2 d postbirth) into 6- to 8-wk-old BALB/c Nude recipients. Nude mice have a mutation in the gene FoxN1, preventing the formation of thymic architecture and resulting in a complete lack of mature T cells. Consistent with a model in which autoimmunity is dependent on an absence of Aire in the medullary stroma, BALB/c Nude recipients of Aire-deficient thymic implants developed lacrimal disease (Fig. 6B, 6C). As expected, disease was not restricted to the lacrimal gland and infiltrates were also observed in the liver, lung, pancreas, prostate, salivary glands, and stomach (data not shown).

T cells from Aire-deficient mice recognize OBP1a

To demonstrate that a lack of OBP1a in the thymic compartment results in a defect in central tolerance, we performed ELISPOT analysis to determine the frequency of autoreactive cells in the

FIGURE 4. CD4+ and CD8+ T cells and B cells are present in the immune infiltrates. A. The lacrimal glands of 6-wk-old Aire-sufficient and Aire-deficient NOD animals were analyzed by immunostaining of frozen sections with Abs specific for CD4 and CD8 (T cells) or IgD (B cells) (original magnification ×20). B. Flow cytometry was also used to determine the relative composition of immune cells in the infiltrate. Numbers represent the percentage of cells within the lymphocyte gate that are surface marker positive. Data are representative of at least five independent experiments.

FIGURE 5. CD4+ T cells are important in the disease process. To determine what cells were capable of transferring disease, cell populations derived from 6- to 8-wk-old NOD, Aire-deficient animals and depleted of either CD4, CD8, or B cells using rabbit complement were injected into immunodeficient NOD.scid hosts. Recipient mice were analyzed for the presence or absence of immune infiltrates 40 d post transfer. A, H&E stained sections were used to assess disease (original magnification ×20). B, Disease scores are shown for individual animals in each group. Data are representative of two independent experiments.
draining cervical LNC of wild-type and Aire-deficient mice. CD4+ T cells were isolated from 10- to 15-wk-old BALB/c Aire-sufficient or Aire-deficient animals and cultured with irradiated APCs loaded with recombinant OBP1a-MBP or MBP tag alone. The number of cells that produced IFN-γ when stimulated with OBP1a was significantly increased in Aire-deficient mice (Fig. 7A), suggesting the release of autoreactive cells from the Aire-deficient thymus and their expansion in the periphery. To further demonstrate that OBP1a-reactive cells were present in the immune cells infiltrating the target organ, CD4+ T cells were purified from the lacrimal glands by flow cytometry and stimulated as described previously (Fig. 7B). Because of the heterogeneity in the number of OBP1a-specific cells present in Aire-deficient mice, this result did not achieve statistical significance ($p = 0.2$). However, three of the four Aire-deficient mice had OBP1a-specific cells present in their lacrimal glands, whereas none of the Aire wild-type mice had OBP1a-specific cells present.

To further demonstrate that OBP1a-specific cells can generate lacrimal autoimmunity, we stimulated cervical LNCs from 6-wk-old NOD Aire-deficient mice in vitro with either OBP1a-MBP fusion protein or MBP fusion protein alone. After 4 d of stimulation, cells were purified by Ficoll centrifugation and 1.5 million cells per mouse were transferred into 6- to 8-wk-old NOD.scid immunodeficient recipients. On transfer into immunodeficient hosts, lacrimal disease in mice that received OBP1a stimulated cells was significantly more severe than in MBP stimulated cells (Fig. 7C). As a control, disease severity in the remaining organs was equivalent in the two groups (Fig. 7D), indicating that stimulation of the cells with OBP1a specifically exacerbated disease only in the lacrimal gland. Paraffin-embedded sections were also stained with anti-CD3 Abs to confirm the presence of T cells in the lesions (Supplemental Fig. 3).

**Discussion**

In this study, we have demonstrated that Aire-deficient mice represent a model for autoimmune dry eye and have further characterized the autoimmune response. Of note, previous work in the model has suggested that the Aire-deficient autoimmune response to the salivary and lacrimal glands is directed against the known autoantigen α-fodrin (7). To carefully assess the Ags potentially targeted in the model, we performed immunoblot analysis with sera from multiple NOD and BALB/c Aire-deficient mice on lacrimal gland extracts and identified a novel molecular target of 18 kDa that proved to be OBP1a. Interestingly, OBP1a is
are shown for individual animals in each group.

from the cervical LNCs of 6-wk-old NOD Aire-deficient mice (n = 5) and Ai
scored for disease severity (original magnification ×10). Disease scores are shown for individual animals in each group.

expressed in the thymus in an Aire-dependent fashion, suggesting that de
defective thymic expression of OBP1a may play a role in the spontaneous dacryoadenitis that develops in the Aire-deficient mouse model.

Previous work on the Aire-deficient model has suggested that α-fodrin is a likely prominent autoantigen associated with SS; however, our data provides evidence for other potential autoantigen
targets. In our analysis we screened for autoantibodies using immu
moblots of whole lacrimal gland extracts. These extracts had detectable α-fodrin present on lacrimal immunoblots. However, no reactivity to α-fodrin was observed when these extracts were probed with Aire-deficient sera. Our inability to detect α-fodrin could be for several reasons. One possibility is that our Aire-deficient mice harbor low titer α-fodrin autoantibodies that are not detectable in our immunoblot assay. The previous study that had identified α-fodrin as an autoantigen in the Aire-deficient model used recombinant protein on an immunoblot and an ELISA to detect reactivity. In our broad autoantibody screen of lacrimal extract immunoblots, reactivity to an 18 kDa Ag was noted in sera of multiple Aire-deficient mice and this Ag was identified as OBP1a. OBP1a is highly expressed in lacrimal gland tissue and has an expression pattern consistent with that of a tissue-restricted Ag. In addition, we also demonstrated that OBP1a is thymically expressed in an Aire-dependent manner. Consistent with a defect in the sele
ction of the T cell repertoire against this Ag, we also have demonstrated that Aire-deficient mice have a higher frequency of OBP1a-specific T cells than wild-type control mice of similar age. Finally, cells derived from Aire-deficient mice and stimulated in vitro with OBP1a are capable of transferring lacrimal disease.

These results have potential implications for our understanding of the Aire-deficient mouse model. There have now been several studies showing structural and cellular changes in the thymus of Aire-deficient mice and there is the suggestion that the immune response in Aire-deficient mice could also target self-Ags whose expression is not thymically regulated by Aire (7, 26, 33). In fact, α-fodrin has been cited as a potential self-Ag that would fit this model as its thymic expression is not Aire-dependent and Aire-deficient mice develop autoantibodies to it (7). In contrast, previous work by our group and the Mathis group has demonstrated that Aire-deficient mice develop autoantibody responses to self-Ags that are thymically expressed in an Aire-dependent manner (20, 32). Further, our group has determined that increased T cell precursor frequencies are present for a retinal autoantigen, IRBP, in the model. Likewise, we demonstrate that in addition to autoantibodies to OBP1a, there is also a detectable increase in the T cell response to OBP1a consistent again with a possible thymic selection defect for this self-Ag. Further study will be needed to determine the relative contribution of the α-fodrin and OBP1a autoimmune responses to the dacryoade
nitis phenotype in the Aire-deficient model, but our data suggest that other Ags besides α-fodrin may contribute to the pathological re
sponse to the lacrimal gland.

Despite the prevalence of several rodent models for SS, only one lacrimal gland specific autoantigen was identified prior to this study (34–39). Autoantibodies to Ro/SSA and La/SSB as well as other ubiquitous nuclear autoantigens have been identified and/or are used in the diagnostic criteria for SS, but reactivity is not exclusive to SS. As outlined above, autoreactivity to another ubiquitous Ag, α-fodrin, has been demonstrated in SS patients (8), as well as nu
merous mouse models for SS (5) including Aire-deficient mice. It is important to note that autoantibodies to α-fodrin have been dem
strated in a number of diseases, including juvenile rheumatoid arthritis, lupus erythematosus without secondary SS, multiple sclerosis, Moyamoya, and glaucoma (10–13, 40). In contrast to these ubiquitously expressed autoantigens, OBP1a expression ap
pears to be restricted mainly to the lacrimal gland providing some evidence for tissue specificity of the autoimmune response.

In addition to a lack of identified autoantigens in these mouse models, the exact mechanisms involved in the pathogenesis of disease are unclear. For example, in the NOD mouse model of SS the organs targeted are gender-dependent, the disease is not ob
served until 12–15 wk of age and is of mild severity compared with what we observe in Aire-deficient mice (34), and autoim
munity may be complicated by the presence of insulin-dependent diabetes. MLR/lpr mice develop lacrimal and salivary gland infiltrates and autoantibodies to the ubiquitous Ro and La Ags but also have generalized lymphoproliferation not observed in SS patients (35–37). More recently, Id3-deficient mice have been shown to have decreased glandular function and lymphocytic infiltrates (41, 42); however, Id3 is also expressed in the target tis
sues and the relevance of this is not clear. In addition, human studies on SS patients have yet to identify a role for Id3 in disease (38). Other models, like the NFS/sld, require the removal of the thymus 3 d postbirth (39). In contrast, the Aire-deficient mouse
model has been clearly demonstrated to have a mechanistic link to thymic T cell selection, is a spontaneous disease process, and has a human correlate in APS1. Although no single mouse system is a perfect model for SS, the Aire-deficient mouse will be a useful tool to study the consequences of a breakdown in immune tolerance to the lacrimal gland.

The role of B cells in this disease process is controversial. Although autoantibodies to OBP1a helped identify the autoantigen involved in this disease process, the transfer of B cell-depleted populations into NOD.scid mice had no appreciable effect on disease induction. In addition, previously published data from our group has shown that passive transfer of sera from Aire-deficient mice is insufficient to cause disease and that genetically deleting B cells from the immune repertoire in Aire-deficient Igα-deficient mice has no effect on the disease process (29). In an intact mouse, it is possible and likely that B cells play a role in Ag presentation in the draining LNCs or target organ (30). However, their role in this process is redundant as removing this cell population did not affect the disease outcome.

Does central tolerance play a role in SS? Some human patients with mutations in AIRE develop KCS and SS, suggesting that there may be a link between AIRE and SS. Furthermore, the work here identifying a novel lacrimal gland-specific autoantigen suggests a potential link between SS and thymic expression of lacrimal gland-specific Ags. Thus, we suggest that a similar unbiased approach to identifying autoantigens in patients with APS1 and/or SS may identify additional autoantigens with clinical relevance. Although OBP1a is a murine gene with no known homolog in humans, it is a member of the lipocalin family which has been implicated in SS (43). Additional work needs to be conducted in human patients to determine whether there is a human homolog or whether a different gene is being targeted. The identification of autoantigens like OBP1a could provide the framework to induce Ag-specific tolerance. It is also important to note that such a treatment approach could thus be feasible for SS patients.

Importantly, both Aire-deficient mice on both the NOD/LtJ and BALB/c backgrounds develop lacrimal gland autoimmunity consisting of immune infiltrates in the target tissue and autoantibodies specific for the lacrimal gland protein OBP1a. Of note, the onset of ocular surface symptoms is stronger in NOD/LtJ mice than BALB/c mice, comparing the two strains there is a difference in onset, severity, and penetrance of ocular surface changes. However, the severity and penetrance of lacrimal gland infiltrates is identical in the two strains (onset is delayed in BALB/c mice as compared with NOD, however). This suggests that lacrimal gland inflammation plays an important role in the ocular surface changes seen in Aire-deficient mice, however, other genetic loci play a key role in the ultimate ocular phenotype. Thus, other genes may be involved in relative susceptibility or resistance to corneal damage and dysfunction.

Finally, our data support the notion that the SS-phenotype in Aire-deficient mice may be driven by autoantigen other than α-fodrin and that a reassessment of the role of α-fodrin in Aire-deficient mice may be helpful. Mechanistic studies similar to our work on IRFB using autoantigen-deficient animals (20) will be required to further clarify the relative contribution of OBP1a and α-fodrin to autoimmunity against the lacrimal gland in this model. Despite this unresolved issue, a clearer picture of the Ag specificity in the Aire-deficient mouse model is beginning to emerge.

Acknowledgments
We thank Jeff Bluestone, Abul Abbas, and T. Shum for critical review of the manuscript, members of the Anderson Laboratory for helpful discussions, and the University of California-San Francisco Biomolecular Resource Center Mass Spectrometry Facility for mass spectrometry.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S1 – BALB/c Aire-deficient mice generate spontaneous dacryoadenitis.

Histological analysis of 15 week old BALB/c animals demonstrates an immune infiltrate in the lacrimal glands of Aire-deficient animals that is not present in Aire-sufficient animals.

Figure S2 – CD4+ and CD8+ T cells and B cells are present in the immune infiltrates of BALB/c Aire-deficient mice.

The lacrimal glands of 12 week old Aire-deficient (A) and Aire-sufficient (B) BALB/c animals were analyzed by immunostaining of frozen sections with antibodies specific for CD4 and CD8 (T cells) or IgD (B cells).

Figure S3 – CD3+ T cells are present in the immune infiltrates of NOD.scid mice that receive OBP1a stimulated T cells.

Cervical lymph node cells harvested from 6 week old NOD.Aire-deficient animals were cultured with 50ug/ml of maltose binding protein (MBP) protein alone (A) or OBP1a-MBP fusion protein (B). After 4 days in culture, lymphocytes were purified by Ficoll centrifugation and 1.5 million cells were adoptively transferred into NOD.scid recipients. Animals were sacrificed 4 weeks post-transfer and paraffin sections were stained with antibodies for anti-CD3 or isotype control.