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

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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.0902434.4.DC1

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Sjögren’s Syndrome (SS) is a human autoimmune disease characterized by immune-mediated 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. 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 lymphoctic 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
Animals were rested comfortably for 5 min, then tear fluid was collected from mice 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 SMZ2000 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; 1–1, 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 Air-deficient and Air-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 (Sigma-Aldrich, St. Louis, MO) via i.p. injection. Animals were rested comfortably for 5 min, then tear fluid was collected via the application of 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.

Immunopurification

Immunopurification of autoantigens was performed using protein agarse G-coupled to Air-deficient 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 agarse G-coupled to Immunoaffinity purification of autoantigens was performed using protein agarose G-coupled to

Immunoaffinity purification

Maltose fusion protein purification

Maltose binding protein (MBP) constructs, fusion protein was prepared by lysing the bacteria with sonication, followed by centrifugation at 8000 g for 10 min, then washed with 30 ml 0.1 M DTT, 10% glycerol, 60 mM Tris. Recombinant MBP was precipitated by adding 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 cocrystallized in a matrix of α-cyan-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 and were reverse transcribed into cDNA using Superscript III (Invitrogen) and oligo-dT primers.

Amplification of OBP1a and cyclophilin from organs

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

Expression 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 OBP1a were used to generate ampiclons containing the full length sequence with restriction sites for subcloning. For pGEX-3X, primers used were: forward primer 5'-CGGATCCGGGAATTCTCTGC-3' and reverse primer 5'-ATATGTTTACCGGCGACGTTCAGTAAAT-3'. These primers incorporated a BamHI site on the 5' end and a NotI site on the 3' end of the ampiclon for subcloning. For pMAL-C2X, primers used were: forward primer 5'-TAGGATCCGGGAAAATTTCTGTCG-3' and reverse primer 5'-TCTGTCGTCTAGTACGACGAGTAAT-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-l 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, Sand Diego, CA). Briefly, cultures of BL-21 bacteria transformed with OBP1a-GST were pelleted and resuspended in 1× 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 + 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.

Maltoolose 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
Preparation and flow cytometry of lymphocytes 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 × 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-sufficient mice were removed and stained with Histopaque (Sigma). Cells were suspended in DMEM medium and 100 μl of the suspension was added to each well of a 96-well plate. Plates were incubated overnight at 37°C. The next day, 500 μl of RPMI 1640 complete medium was added to each well. The plates were incubated for 24 h at 37°C. The effector CD4+ T cells and irradiated APC (5000 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. 5551216, 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. 5551216, BD Pharmingen, San Diego, CA) and incubated overnight at 4°C. After further washout 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 Transres 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.

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 dispensed via pipetting, recovering 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'-GAAGAAGAGATGAAAGTCACAT-3' and Reverse-5'-ATCTTCTTGCAAATACCCAGTGATT-3'; Probe-5'-GAATGCAAGGAAATGAAAGTCACAT-3'; Reverse-5'-ATTCCTTTGCAAAATACCAAGTCTGAT-3'; Probe-5'-FAM-TCAATGGA- AAATGGCACAGTGCTTATGACCA-3'-TAMRA. For Spt1, the following primers were used: Forward-5'-GCTTGTGGTGGCTACTATCTGCTGCTC-3'; Reverse-5'-AATCAGCAATGTCCAAGTATGTCGACGTTCGACCCA-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 μM 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 cornalian 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 dissolating 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 autoimmune 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 Aire-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 immunity to OBP1a from whole eye extracts represented a contamination of the eye extract preparation with immunohistochemistry with sera derived from Aire-deficient mice and Aire-sufficient animals (original magnification ×40). C, NOD.scid lacrimal gland extracts prepared in Laemmli sample buffer and immunoblotted with sera derived from 6- to 8-wk-old NOD.Aire-deficient (individual animals listed by number) and Aire-sufficient (Wt) animals target an ∼18 kDa protein. D, Reactivity to the 18 kDa Ag is not limited to the NOD background and Aire-deficient BALB/c mice (individual animals listed by number) also show 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-wk-old NOD:Aire-deficient animals or three 10-wk-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 Aire-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 immunohistochimistry 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 Aire-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 1. 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>

**FIGURE 2.** The lacrimal gland is a target of autoimmunity and expresses an 18 kDa autoantigen. A, Histological analysis of 6-wk-old NOD:Aire-sufficient (+/+) and Aire-deficient (-/-) animals demonstrated a profound immune infiltrate in the lacrimal glands of Aire-deficient animals absent from Aire-sufficient animals (original magnification ×40). B, NOD.scid lacrimal gland sections were embedded in OCT, sectioned, and used to perform indirect immunohistochemistry with sera derived from Aire-deficient mice and Aire-sufficient animals (original magnification ×40). C, NOD.scid lacrimal gland extracts prepared in Laemmli sample buffer and immunoblotted with sera derived from 6- to 8-wk-old NOD:Aire-deficient (individual animals listed by number) and Aire-sufficient (Wt) animals target an ∼18 kDa protein. D, Reactivity to the 18 kDa Ag is not limited to the NOD background and Aire-deficient BALB/c mice (individual animals listed by number) also show 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-wk-old NOD:Aire-deficient animals or three 10-wk-old NOD:Aire-sufficient controls.
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 this 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 (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.

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

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**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
expressed in the thymus in an *Aire*-deficient fashion, suggesting that defective thymic expression of OBPla 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 immunoblots 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 OBPla. OBPla 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 OBPla is thymically expressed in an *Aire*-dependent manner. Consistent with a defect in the selection of the T cell repertoire against this Ag, we also have demonstrated that *Aire*-deficient mice have a higher frequency of OBPla-specific T cells than wild-type control mice of similar age. Finally, cells derived from *Aire*-deficient mice and stimulated in vitro with OBPla 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 OBPla, there is also a detectable increase in the T cell response to OBPla 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 OBPla autoimmune responses to the dacryoadenitis phenotype in the *Aire*-deficient model, but our data suggest that other Ags besides α-fodrin may contribute to the pathological response 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 numerous mouse models for SS (5) including *Aire*-deficient mice. It is important to note that autoantibodies to α-fodrin have been demonstrated 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, OBPla autoimmune expression appears 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 observed until 12–15 wk of age and is of mild severity compared with what we observe in *Aire*-deficient mice (34), and autoimmunity 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 tissues 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 1) b 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 autoantigens 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 IRBP 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.

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