IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Multiple Lesions Contribute to Infertility in Males Lacking Autoimmune Regulator

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Male factors, including those of autoimmune origin, contribute to approximately 50% of infertility cases in humans. However, the mechanisms underlying autoimmune male infertility are poorly understood. Deficiency in autoimmune regulator (*AIRE*) impairs central immune tolerance because of diminished expression of self-antigens in the thymus. Humans with *AIRE* mutations and mice with engineered ablation of *Aire* develop multiorgan autoimmunity and infertility. To determine the immune targets contributing to infertility in male *Aire*-deficient ($-/-$) mice, *Aire* $-/-$ or wild-type (WT) males were paired with WT females. *Aire* $-/-$ males exhibited dramatically reduced mating frequency and fertility, hypogonadism, and reduced serum testosterone. Approximately 15% of mice exhibited lymphocytic infiltration into the testis, accompanied by atrophy, azoospermia, and reduced numbers of mitotically active germ cells; the remaining mice showed normal testicular morphology, sperm counts, and motility. However, spermatozoa from all *Aire* $-/-$ mice were defective in their ability to fertilize WT oocytes *in vitro*. Lymphocytic infiltration into the epididymis, seminal vesicle, and prostate gland was evident. *Aire* $-/-$ male mice generated autoreactive antibodies in an age-dependent manner against sperm, testis, epididymis, prostate gland, and seminal vesicle. Finally, expression of *Aire* was evident in the seminiferous epithelium in an age-dependent manner, as well as in the prostate gland. These findings suggest that *Aire*-dependent central tolerance plays a critical role in maintaining male fertility by stemming autoimmunity against multiple reproductive targets. (*Am J Pathol* 2021, 191: 1592–1609; <https://doi.org/10.1016/j.ajpath.2021.05.021>)

Infertility affects up to 25% of reproductive-age couples worldwide.^{1,2} Historically, compromised fertility has most often been attributed to female pathologies, and the range of treatment options currently available reflects this bias. However, it is now understood that roughly one-half of all infertility cases are due to male-specific factors.³ Many cases of male infertility are idiopathic, but known causes of male infertility include pregonadal endocrine, genetic, or coital disorders; gonadal dysfunction involving spermatogenesis arrest, cryptorchidism, or varicocele; and post-gonadal problems, including sperm blockage or genital infection.^{4,5}

The immune system is responsible for controlling the potentially damaging influence of infections, while maintaining tolerance to self-antigens. Failure of the latter manifests as autoimmune disease, often involving complex genetic and environmental interactions.⁶ As many as 10% of

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the known causes of male-factor infertility involve immune conditions, which include orchitis, epididymitis, and autoantibody targeting.⁷ Thus, infection or damage to the male reproductive tract can result in immune targeting of antigens unique to germ cells, seminiferous epithelium, the epididymis, and/or the vas deferens.^{8,9}

Monogenic autoimmune diseases, which result from single-gene deficiencies, are rare, but have provided insight into the mechanisms of establishment and breakdown of self-discrimination/non-self-discrimination.¹⁰ Autoimmune polyendocrine syndrome type I (APS-1) is one such disease that results from function-disrupting mutations in the autoimmune regulator (*AIRE*) gene.^{11,12} APS-1 is characterized by high serum titers of autoantibodies and is diagnosed on the basis of the presence of two of three pathologies of principle targets: adrenal insufficiency, chronic mucocutaneous candidiasis, and hypoparathyroidism.^{13–15} APS-1 also targets other systems, including male and female reproductive organs, often leaving patients infertile.¹⁴

AIRE is a 58-kDa nuclear glycoprotein that is most prominently expressed in medullary thymic epithelial cells (mTECs) and possesses structural and functional attributes suggestive of a transcription factor.^{16,17} *AIRE* has a vital role in establishing central immune tolerance by virtue of its ability to induce promiscuous expression of numerous antigens in mTEC that are otherwise restricted to one or a few other tissues.¹⁷ This occurs, in part, through one of *AIRE*'s two plant homeodomain motifs, which targets *AIRE* to genes with histone markers typical of transcriptionally inactive genes.^{18–20} Once expressed, these antigens are processed and presented in the context of surface major histocompatibility molecules, either directly by thymic epithelial cells or indirectly via cross-presentation by thymic dendritic cells. T cells encountering these antigens via their cognate T-cell receptors are either directed toward the regulatory T-cell lineage or deleted and thereby prevented from entering the periphery where they may otherwise cause autoimmune disease.^{21–24}

In mice, targeted deletion of *Aire* (*Aire*^{−/−}) reproduces many key features of APS-1. mTECs of *Aire*^{−/−} mice have a significantly altered self-antigen expression profile, which allows inappropriate development and emigration of self-reactive T cells from the thymus and culminates in destructive autoimmune response mediated by T cells and serum autoantibodies.^{17,25–27} In addition, disease onset and severity are highly variable both within and between genetic backgrounds.²⁸ Among *Aire*-regulated antigens are those found within, and even exclusive to, the male reproductive system.¹⁸ Interestingly, several authors have noted reduced fertility in male *Aire*^{−/−} mice, as well as autoimmune prostatitis.^{17,25–27,29} However, the lesion leading to infertility in male *Aire*^{−/−} mice has not been completely identified. In the present study, the consequence of a disruption of *Aire* in male mice for fertility were explored, examined in the potential targets of autoimmune disease of the male

reproductive tract were examined in detail, and expression of the *Aire* gene was assessed in key reproductive tract organs.

Materials and Methods

Animals

Mice were housed under pathogen-free conditions under a 12-hour light:12-hour dark photoperiod at the University of Kansas Medical Center or Michigan State University Campus Animal Resources and were provided with sterile food and water ad libitum. Experiments with animals complied with NIH's *Guide for the Care and Use of Laboratory Animals*³⁰ and were approved by the Institutional Animal Care and Use Committee at University of Kansas Medical Center and Michigan State University. Recombinase activating gene 2 (Rag2)-deficient mice [Rag2^{−/−}; C.129S6(B6)-Rag2^{tm1Fwa}] were purchased from Taconic (Rensselaer, NY). *Aire*^{−/−} mice on the Balb/cJ genetic background (more than eight generations)^{17,28} were donated by Christophe Benoist (Harvard Medical School, Boston, MA), and were maintained by breeding heterozygotes. *Gt(ROSA)26Sor*^{tm4(ACTB-tdTomato,-EGFP)Luo/J} (mTmG)³¹ and Balb/cJ wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Aire-Cre* mice were generated as described,³² and genotypes for all mice were determined by PCR, as previously described.^{32–34}

Fertility Assessment

Fertility was evaluated in 6- to 13-week-old *Aire*^{−/−} males and WT controls. WT or *Aire*^{−/−} males were cohabitated singly with WT females (6 to 10 weeks old), which were checked each morning until a copulation plug was detected, at which time the male was euthanized for blood and tissue collection. The females were allowed 23 days postcopulation to deliver pups, and the offspring were counted if a litter was produced. If a copulation plug was not visually confirmed over a period of 20 days (approximately four ovarian cycles), the male was euthanized, and the female was permitted 21 days of isolation to verify absence of pregnancy before being mated to a proven WT BALB/c male to confirm the female's fertility.

Blood and Tissue Collection

Mice were anesthetized with a sterile solution of Avertin (2,2,2-tribromoethanol dissolved in 2-methyl-2-butanol; 250 mg/kg intraperitoneally; Sigma-Aldrich, St. Louis, MO). Serum was collected under anesthesia via cardiac puncture, after which mice were euthanized via cervical dislocation and bilateral pneumothorax. Testis, epididymis, seminal vesicles, and prostate gland were removed and weighed, fixed in 4% paraformaldehyde overnight, and embedded in paraffin for histopathologic analysis.

Determination of Serum Hormone Levels

Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA). FSH and testosterone were quantified by radioimmunoassay, with ranges of 2.0 to 25 ng/mL and 0.1 to 10 ng/mL, respectively. LH was measured by sandwich radioimmunoassay, with an assay range of 0.07 to 37.4 ng/mL. CVs for the assays were as follows: FSH, 6.9% (intra-assay) and 9.4% (interassay); LH, 4.5% (intra-assay) and 8.3% (interassay); and testosterone, 4.7% (intra-assay) and 8.5% (interassay). Standards for the assays included a reference preparation of mouse LH (AFP5306A) and FSH (AFP5308D); further details of all assays performed are available (<https://med.virginia.edu/research-in-reproduction/ligand-assay-analysis-core>, last accessed May 18, 2021).

Caudal Sperm Production and Analysis

Caudal epididymides were excised from mice following sacrifice, rinsed, weighed, and transferred to 750 μ L of Human Tubal Fluid media (EMD Millipore, Burlington, MA). The tissue was bisected, and sperm was allowed to exude for 1 hour at 37°C. Sperm count was determined using a hemocytometer and normalized to the weight of the tissue.

Sperm Motility Analysis

Sperm motility analysis was performed as previously described.³⁵ Briefly, 3×10^6 cells were resuspended in modified Tyrode medium containing 1.7 mmol/L CaCl_2 . The cells were labeled with the green fluorescent nucleic acid stain SITO21 and loaded onto glass cell chambers (Leja Products BV, Nieuw-Vennep, the Netherlands). The chambers were viewed using an Olympus BX51 microscope (Olympus, Feasterville, PA) while being maintained at 37°C on a heated platform. Samples were analyzed by computer-assisted sperm analysis using the Minitube SpermVision Digital Semen Evaluation system version 3.5 (Penetrating Innovations, Verona, WI). Sperm motility parameters, including total and progressive motility, were measured.

Sperm Production Analysis

Daily sperm production (DSP) was determined as previously described.³⁶ One testis was frozen in liquid nitrogen and stored at -80°C until analysis. To determine DSP, testes were thawed, decapsulated, weighed, and dissolved in 1 mL saline containing 0.01% Triton X-100 by homogenization (Tissue-Tearor, model 985370-395; BioSpec Products Inc., Bartlesville, OK) at 15,000 rpm for 3 minutes. Elongated spermatids (stages 14 to 16), which are resistant to homogenization, were counted by trypan blue dye

staining on a hemocytometer. Total spermatid number per testes was divided by weight of the decapsulated testis to give spermatids per gram of testis. Developing mouse spermatids spend approximately 4.84 days in steps 14 to 16 during spermatogenesis.³⁷ Thus, the values for spermatids/testis and spermatids per gram testis were divided by 4.84 to obtain the DSP and efficiency (DSP per gram) of sperm production, respectively.

Histopathologic Evaluation and Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded reproductive tract tissues were sectioned at a thickness of 5 μ m and stained with hematoxylin and eosin (Dako, Santa Clara, CA). Immunohistochemistry was used for the detection of CD3-positive and MKI67 (Ki-67)—positive cells within the tissues of the male reproductive tract of *Aire*^{-/-} and WT controls. To this end, sections were rehydrated through decreasing concentrations of ethanol and, for CD3 and Ki-67 staining, subjected to antigen retrieval using Reveal buffer (BioCare Medical, Pacheco, CA). Tissue sections were then blocked in 10% goat serum and incubated overnight at 4°C with monoclonal rabbit anti-mouse CD3 (clone SP7; 1:100 dilution of neat supernatant; Abcam, Cambridge, UK) or rabbit anti-human Ki-67 (clone SP6; 1:200 dilution of supernatant; Thermo Fisher, Waltham, MA). The SP7 antibody recognizes the CD3 ϵ chain of the CD3 complex on T cells and has been validated by the manufacturer via Western blot analysis, flow cytometry, and immunohistochemistry using CD3⁺ T-cell lines. The Ki-67 antibody has been validated by the manufacturer using cell starvation/replacement treatment and by analysis of knockout cells. In our experiments, isotype-matched rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used as a negative control. Sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; catalog number BA-1000), and subsequently depleted of endogenous peroxidases. For colorimetric detection of bound antibodies, streptavidin peroxidase and aminoethyl carbazole were added to the sections, producing a red-brown deposit (Invitrogen, Carlsbad, CA). Sections were counterstained with hematoxylin and viewed by bright-field microscopy on a Nikon 80i microscope (Nikon, Melville, NY). To assess mean numbers of mitotically active germ cells in seminiferous tubules, testis sections were stained for Ki-67. Ki-67—positive cells were counted in five random fields at $\times 200$ magnification. Data collected included the average numbers of Ki-67—positive cells per tubule, as well as the overall proportion of tubules that contained Ki-67—positive cells.

Immunoblotting

WT and *Aire*^{-/-} mouse sera were used to probe testis, caput, cauda, seminal vesicle, prostate, and sperm lysates

from Rag2^{-/-} mice for the presence of autoantibodies. Tissue was homogenized on ice in radio-immunoprecipitation assay buffer and boiled for 5 minutes in reducing buffer. Tissue lysate (400 µg) was run on a 4% to 20% TGX stain-free curtain gel (BioRad, Hercules, CA) and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Chicago, IL) at 100 V for 20 minutes. The membrane was blocked for 3 hours at room temperature in 3.5% bovine serum albumin in phosphate-buffered saline before being placed inside a Mini-Protein II Multiscreen apparatus (BioRad). Sera (1:750 dilution) from WT and *Aire*^{-/-} mice were loaded into the slots and incubated at 4°C overnight. Membranes were washed in phosphate-buffered saline containing 0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich). Immunoreactivity was visualized using an ECL detection kit (GE Healthcare Life Sciences), according to the manufacturer's instructions.

Immunofluorescence

Cellular reactivity with serum autoantibodies was determined by immunofluorescence using sera from *Aire*^{-/-} mice to probe testis, epididymis, seminal vesicles, and prostate glands of Rag2^{-/-} mice. Briefly, tissues were flash frozen in dry ice-cooled 2-methylbutane (Sigma-Aldrich) for 5 minutes before embedding in OCT cryoprotective medium (Sakura Finetek, Torrance, CA). Sections were cryosectioned (10 µm thick) and fixed in 100% cold acetone for 5 minutes. Serum from WT and *Aire*^{-/-} mice was added at a 1:40 dilution, and bound antibodies were visualized using a goat anti-mouse IgG fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch). To determine whether endogenous autoantibodies were bound within the cauda epididymis, fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody was applied directly onto cryosections from WT and *Aire*^{-/-} mice. All sections were coverslipped using Prolong Gold containing DAPI (Invitrogen) and viewed on a Nikon 80i fluorescent microscope.

Expression of *Aire* in the reproductive tract was evaluated, as previously described,³³ using a commercially available primary antibody (rat anti-mouse *Aire*; clone 5H12; 5 µg/mL; Thermo Fisher). Specificity of this antibody was confirmed by us previously³³ using thymus tissue from WT and *Aire*^{-/-} mice as positive and negative controls, respectively. WT males were euthanized at 3 and 12 weeks of age, and testis, epididymis, prostate, and seminal vesicles were fixed in 4% paraformaldehyde, cryopreserved in 30% sucrose, and frozen in OCT embedding medium. Tissues were sectioned at 5 µm thickness, permeabilized with 0.1% Triton X-100, and blocked in 10% goat serum. Sections were then incubated in primary antibody at 4°C overnight, washed, and incubated with goat anti-rat secondary antibody conjugated to Alexa Fluor 488 (10 µg/mL; Thermo Fisher; catalog number A11006). Sections were

counterstained with DAPI (Vector Laboratories) and imaged as above.

In Vitro Fertilization

Female WT mice were given pregnant mare's serum gonadotropin (5 IU/mL intraperitoneally), followed by human chorionic gonadotropin (5 IU/mL intraperitoneally) 48 hours later. Twelve hours after human chorionic gonadotropin injection, the mice were sacrificed and the oocytes were flushed from the oviduct, washed through three microdrops of FHM-HEPES (EMD Millipore), and placed in a culture dish with Human Tubal Fluid media. To collect sperm, WT or *Aire*^{-/-} males were sacrificed on the same day as oocyte collection, and caudal epididymis was dissected and incubated at 37°C in 1 mL of Human Tubal

Figure 1 Fertility parameters in *Aire*^{-/-} male mice. Wild-type (WT) control (+/+) and *Aire*^{-/-} male mice were paired with +/+ females, which were monitored for copulation plugs for up to 21 days. **A:** Percentage of +/+ controls and *Aire*^{-/-} males that produced a copulatory plug. **B:** Percentage of +/+ and *Aire*^{-/-} males that generated viable litters. **C:** Numbers of offspring produced by +/+ and *Aire*^{-/-} males. Each data point represents the litter size of an individual male; gray horizontal bars represent means. *n* = 10 WT mice (**A–C**); *n* = 11 *Aire*^{-/-} mice (**A–C**). **P < 0.01.

Fluid for at least 90 minutes. Capacitated sperm (1 to 2×10^6) was incubated with collected oocytes at 37°C for an additional 4 to 6 hours. Oocytes were then washed and cultured in microdrops of potassium-supplemented simplex-optimized medium (KSOM) with amino acids and D-glucose (EMD Millipore). Embryos were visualized and staged using an inverted light microscope 24 and 96 hours after fertilization.

Bioinformatic Identification of Testis-Specific Aire Target Genes

To identify tissue-specific gene sets, we combined the genome-wide RNA-sequencing data from two previous studies that cover 47 diverse tissues, including testis, thymus, uterus, brain, adrenal gland, liver, placenta, ovary, and kidney.^{38,39} Quantile normalization was applied to normalize the data across different samples and remove potential confounding factors. This was followed by hierarchical clustering to visualize clusters of tissue-specific genes. To remove housekeeping genes, the entropy of

expression values for each gene across the samples was calculated and ranked. Only the top 50% genes with small entropy values were included in the downstream analyses. For each of these, the z-scores of the gene's expression in each tissue were calculated and z-scores of >2.5 were defined as tissue-specific.

To identify Aire target genes, the published RNA-sequencing data from various populations of murine thymic epithelial cells was used.⁴⁰ Two versions of target genes were generated. For the first, gene expression was compared in *Aire*^{-/-} versus wild-type mTECs. For the second, gene expression was compared in Aire mRNA-negative versus Aire mRNA-positive mTECs, which were identified bioinformatically on the basis of known TEC markers as well as Aire mRNA expression. For differential gene expression analysis, two criteria were used: subtraction of log₂-transformed fragments per kilobase of transcript per million mapped reads values from the two conditions in comparison and fold change of log₂-transformed fragments per kilobase of transcript per million mapped reads values. The genes were then ranked from largest to smallest value

Figure 2 Testicular atrophy and lymphocytic infiltration occur in a subset of *Aire*^{-/-} mice. **A:** Representative examples of testes from wild-type (WT; left panel) and *Aire*^{-/-} (middle and right panels) mice. **B:** Histologic analysis of WT (left panel) and *Aire*^{-/-} (middle and right panels) testis, showing lymphocytic infiltrate and interstitial cells. **C:** Testis weight in +/+ and *Aire*^{-/-} mice. Each data point represents an individual animal; gray horizontal bars represent the mean. Weights of testis were recorded for 34 WT and 34 *Aire*^{-/-} mice. **D:** Enlarged view of lymphocytic infiltrate, boxed area in B. $n = 17$ WT testis (B); $n = 23$ *Aire*^{-/-} testis (B). Scale bars = 100 μm (B and D).

Figure 3 Disruption of seminiferous tubule organization in *Aire*^{-/-} mice. **A:** Representative images comparing Ki-67 immunoreactivity in testis of wild-type (WT) and *Aire*^{-/-} males. **B:** The proportion of seminiferous tubules containing proliferative (Ki-67⁺) germ cells in WT and *Aire*^{-/-} testis, as evaluated by immunohistochemistry. **C:** Ki-67⁺ germ cells per tubule from immunohistochemical stains of WT and *Aire*^{-/-} mice. Data are given as means $\pm$ SEM (**B** and **C**). $n = 3$ WT males (**A**); $n = 5$ *Aire*^{-/-} males (**A**). $**P < 0.01$. Scale bars = 100 μ m. KO, knockout.

based on the two metrics separately, and those ranked at the top 10% on both metrics were considered as down-regulated target genes by Aire, whereas those ranked at the bottom 10% on both metrics are considered as up-regulated targets. The Aire target genes were then compared with different sets of tissue-specific genes to identify tissue-specific Aire target genes, using hypergeometric tests to determine the statistical significance of the overlap between Aire target genes and tissue-specific genes. The overrepresentation of Aire target genes in different tissues was then quantified by $-\log_{10}(P \text{ value})$. Finally, Gene Ontology and pathway enrichment analysis⁴¹ were performed using David Gene Functional Annotation tool.⁴² The top enriched pathways

were used to analyze the potential functional roles of tissue-specific Aire target genes.

Aire Gene Expression in Male Reproductive Tract Tissues

Aire gene expression was evaluated by two methods: quantitative RT-PCR (RT-qPCR) and reporter gene analysis. For mRNA analysis, testis, epididymis, prostate, and seminal vesicles were harvested from 3- and 12-week-old WT males, frozen in liquid nitrogen, and processed for RNA extraction using TRIzol. In brief, frozen tissues were lysed with 1 mL of TRIzol using Omni Bead Ruptor 4 (Omni

Figure 4 Immune cell infiltration into testes of *Aire*^{-/-} mice. **A:** Representative sections of wild-type (WT; left panel) and *Aire*^{-/-} (right panel) testes stained with anti-CD3 antibody. **B:** Representative sections of WT (left panel) and *Aire*^{-/-} (right panel) testes stained with anti-F4/80 (macrophages) antibody. $n = 7$ WT testes (**A**); $n = 20$ *Aire*^{-/-} testes (**A**); $n = 3$ WT testes (**B**); $n = 5$ *Aire*^{-/-} testes (**B**). Scale bars: 50 μ m (**A**); 100 μ m (**B**).

Figure 5 Identification of anti-testes autoantibodies in *Aire*^{-/-} mice. **A:** Indirect immunofluorescence of *Rag2*^{-/-} testes cryosections, probed with serum from wild-type (WT) or *Aire*^{-/-} mice, followed by a fluorescently labeled anti-mouse secondary antibody. Representative sections are shown. **B:** Testicular lysate from a *Rag2*^{-/-} mouse was probed with serum samples from WT and *Aire*^{-/-} mice. Each lane represents an individual mouse; ages in weeks are indicated at the top. Numbers on right, molecular weight marker (kDa). **C:** Direct immunofluorescence of testis from an *Aire*^{-/-} mouse, using a fluorescently labeled anti-mouse IgG antibody to detect endogenously bound autoantibodies. A representative section is shown. *n* = 5 WT mice (**A**); *n* = 12 *Aire*^{-/-} mice (**A**); *n* = 3 (**C**). Scale bars: 50 μm (**A**); 100 μm (**C**). KO, knockout.

International, Kennesaw, GA), then transferred into a phase-lock tube. A total of 0.2 mL of chloroform was directly added, followed by 10 minutes of centrifugation at 12,000 × *g*. The resulting colorless upper aqueous phase was

carefully transferred into a new 1.5-mL Eppendorf tube and washed with 0.5 mL of isopropyl alcohol and 1 mL 75% ethanol. Resulting RNA pellet was dissolved in RNase-free water, assessed for 260/280 ratio using Nanodrop, and then

Figure 6 Reduced testosterone and oligospermia in a subset of *Aire*^{-/-} mice. **A:** Serum samples from wild-type (WT) and *Aire*^{-/-} mice were evaluated for testosterone. **B:** Capacitated epididymal sperm were measured by swim-out assay, and adjusted by caudal weight, of WT and *Aire*^{-/-} mice. **C** and **D:** Daily sperm production (DSP; **C**) and sperm production efficiency (**D**), as measured per gram testis weight, in WT and *Aire*^{-/-} mice. **C** and **D:** Each data point represents an individual mouse, and numbers highlight individual mice of interest. **E:** Proportion of wild-type oocytes that developed to the two-cell and blastocyst stages following *in vitro* fertilization with sperm from WT (+/+) or *Aire*^{-/-} males. Data represent means ± SEM (**A** and **B**). *n* = 8 WT mice (**A**); *n* = 14 *Aire*^{-/-} mice (**A** and **B**); *n* = 11 WT mice (**B**); *n* = 83 oocytes from WT males (**E**); *n* = 102 oocytes from *Aire*^{-/-} males (**E**); *n* = 4 WT and *Aire*^{-/-} males (**E**). *P < 0.05.

Figure 7 Overrepresentation of Aire target genes within tissue-specific gene sets. The hypergeometric test was used to calculate P values to evaluate the enrichment of Aire target genes overlapping with tissue-specific gene sets across 47 tissues. **A:** Aire up-regulated genes. **B:** Aire down-regulated genes. The y axis shows $-\log_{10}(P \text{ value})$; and x axis, 47 tissue-specific gene sets. Two versions of Aire target genes from: $Aire^{-/-}$ versus wild type (red) and Aire negative versus Aire positive (blue). The y-axis value of 1 ($P = 0.1$) is marked by the red dotted line.

converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen, Germantown, MD); catalog number 205314), followed by real-time quantitative PCR (QuantStudio 5; Applied Biosystems, Waltham, MA). Samples were analyzed using the delta delta cycle threshold (ddCT) method for the relative expression of Aire mRNA (TaqMan Probe Assay ID: Mm00477452_g1). *Gapdh* (TaqMan Probe Assay ID: Mm99999915_g1) was used as a housekeeping control.

In addition to mRNA analysis, we generated a transgenic reporter system that indicates expression of *Aire* or a history

thereof. mTmG mice³¹ express a fluorescent reporter construct that allows constitutive expression of membrane-targeted tandem dimer Tomato (mT) in the absence of Cre recombinase. In the presence of Cre, the mT locus is excised, resulting in expression of membrane-enhanced green fluorescent protein (mG). mTmG females were mated with *Aire-Cre* males³² such that all fetuses were heterozygote for both the *mTmG* and the *Aire-Cre* loci, and thus expressed mG in all *Aire*-expressing tissues. *Aire-Cre*/*mTmG* males were sacrificed at 3 and 9 weeks of age, and tissues were harvested, fixed, and sectioned. Sections were

Figure 8 Histologic analysis of caput and cauda epididymis. Representative sections of caput (A and B) and cauda (C and D) epididymis were stained with hematoxylin and eosin (A and C) or antibody against CD3 (B and D). Boxed areas in A and C are shown at higher magnification to the right. $n = 7$ wild-type animals (A–D); $n = 20$ *Aire*^{-/-} animals (A–D). Scale bars: 100 μ m (A and C); 50 μ m (B and D).

coverslipped in medium containing DAPI and imaged on a Nikon Eclipse Ti epifluorescence microscope.

Statistical Analysis

Hormone values, caudal sperm count, Ki-67⁺ cells per seminiferous tubule, and testis weights between WT controls and *Aire*^{-/-} mice were analyzed by two-tailed *t*-test. Percentages of mice capable of mating and producing viable offspring and *in vitro* fertilization results were compared by χ^2 analysis. Outliers in hormone data were identified in two (one WT and one *Aire*^{-/-} mouse) cases using the Grubbs test, and were excluded from analysis. Results were considered significantly different when

$P < 0.05$. All statistical analyses were performed using SigmaStat version 3.5 (Systat, San Jose, CA). All experiments were repeated with a minimum of three biological replicates; n is indicated in the appropriate locations for each experiment in the figure legends.

Results

Assessment of Fertility in Male *Aire*^{-/-} Mice

To examine fertility, WT or *Aire*^{-/-} males were housed with WT females and monitored each morning for the presence of a copulatory plug. All 10 WT males produced a plug within 5 days of pairing with a female,

Figure 9 Antibody reactivity to epididymis in *Aire*^{-/-} mice. **A:** Serum from wild-type (WT) or *Aire*^{-/-} mice was applied to cryosections of epididymides of *Rag2*^{-/-} mice, which were subsequently probed with a fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG secondary antibody. **B:** Epididymal lysate from a *Rag2*^{-/-} mouse was probed with sera from WT and *Aire*^{-/-} mice. Each lane represents an individual mouse; ages in weeks are indicated at the top, and numbers on right represent the molecular weight marker (kDa). **C:** Cryosections of epididymides from WT or *Aire*^{-/-} mice were probed with FITC-labeled anti-mouse IgG; positive signal represents endogenously bound autoantibodies. *n* = 5 WT mice (**A**); *n* = 12 *Aire*^{-/-} mice (**A**); *n* = 2 WT mice (**C**); *n* = 4 *Aire*^{-/-} mice (**C**). Scale bars: 100 μ m (**A**); 50 μ m (**C**).

whereas only 5 of 11 *Aire*^{-/-} males produced a copulation plug (Figure 1A). Those that did, required up to 2 weeks to mate, and plugs were often liquefied. In addition, only 2 of the *Aire*^{-/-} males produced viable litters, in contrast to 8 of the 10 females that mated with WT males (Figure 1, B and C). Average litter sizes of *Aire*^{-/-} and WT males, excluding nonproductive males, were 2.0 ± 1.0 and 5.9 ± 1.5 pups, respectively ($P = 0.1$) (Figure 1C).

Testicular Atrophy and Germ Cell Loss Occur in a Subset of *Aire*^{-/-} Males

Overall, gross morphology and tissue weights were comparable between genotypes, with the exception of several males that had reduced testicular weight compared with WT controls or the remaining *Aire*^{-/-} males (Figure 2, A and C). When matched for age, testis weight did not differ significantly between genotypes. However, of 34 total males

examined, 5 (approximately 15%) had small testis, weighing half or less of the mean testes weight of the other mice within their respective age groups. This included one 12-week-old *Aire*^{-/-} male, two approximately 22-week-old *Aire*^{-/-} males, and two of four 35- to 55-week-old *Aire*^{-/-} males.

Histologic evaluation of the testes revealed differences in the minor subset of males that were affected by testicular atrophy. In these mice, severe reductions in spermatogenic cells were apparent, particularly at the spermatid/spermatozoan stages (Figure 2, B and D). Loss in spermatogenesis appeared to be progressive; in one aging (150-day-old) animal, atrophic tubules and spermatogonia were present in some tubules, whereas other tubules contained Sertoli cells and spermatogonia together with sloughed germ cell tubule lumen and still other tubules had overtly normal histology with a few pyknotic spermatids (Figure 2, B and D). Consistent with this, germ cells displayed a rounded morphology, were displaced from the basal region of the tubule, and had reduced expression of the proliferation marker, Ki-67 (Figure 3). This trend held true even in testis from *Aire*^{-/-} mice without overt evidence of testicular inflammation or atrophy. Lymphatics were often dilated, and there was increased cellularity and lymphocyte infiltration in the interstitial space (Figure 2, B and D).

Immunohistochemistry and immunofluorescence analysis confirmed presence of T cells and macrophages in atrophic testis. CD3⁺ T-cell infiltration was observed in 1 of 20 knockout mice examined (5%), mostly contained within the interstitial space (Figure 4A). Occasional T cells could be identified within the lumen of seminiferous tubules that were devoid of germ cells. In addition, numerous macrophages were found in the interstitial space of an atrophic *Aire* knockout testis (Figure 4B). To determine whether testis- or sperm-reactive antibodies were produced by *Aire*^{-/-} mice, cryosections of testis from *Rag2*^{-/-} mice (used to ensure absence of endogenous antibodies) were probed with sera from WT or *Aire*^{-/-} mice. Of the four mice examined, three had similar reactivity to WT control mice. Serum from one mouse, however, showed strong reactivity to spermatid heads (Figure 5A). The spectrum of possible antigenic targets of testicular cells was examined by probing lysate of *Rag2*^{-/-} testis with serum from 7- to 9-week-old WT or *Aire*^{-/-} males by Western blot analysis. Although sera from 7- to 9-week-old wild-type mice had minimal reactivity to testicular lysate, serum reactivity was apparent in *Aire*^{-/-} mice of this age (Figure 5B). In 4- to 5-week-old mice, serum antibody reactivity against testicular lysate was also present: sera from one of two WT mice, and all three *Aire*^{-/-} mice, examined showed reactivity to testicular lysate. Finally, endogenous binding of autoantibodies was investigated by direct immunofluorescence, probing testis of *Aire*^{-/-} (Figure 5C) or WT mice (data not shown; *n* = 3 per genotype) with fluorescein isothiocyanate-conjugated anti-mouse IgG. Surprisingly, little or no endogenous antibody binding was observed.

Figure 10 Autoimmunity against the seminal vesicle of *Aire*^{-/-} mice. **A** and **B**: Representative sections of seminal vesicle from wild-type (WT) or *Aire*^{-/-} mice were stained with hematoxylin and eosin (**A**) or antibody against CD3 (**B**). **Boxed area** in **A** is shown at higher magnification to the right. **C**: Seminal vesicle lysate from a *Rag2*^{-/-} mouse was probed with sera from WT and *Aire*^{-/-} mice. Each lane represents an individual mouse; ages in weeks are indicated at the top, and numbers on right represent the molecular weight marker (kDa). *n* = 7 WT mice (**A** and **B**); *n* = 19 *Aire*^{-/-} mice (**A** and **B**). Scale bars: 100 μ m (**A**); 50 μ m (**B**).

Infertility in *Aire*^{-/-} Mice Is Associated with Disruptions in Testosterone Production and Spermatogenesis and Testicular Autoimmunity

The histologic findings of the testis led us to ask whether steroidogenesis and/or spermatogenesis were impaired in mice lacking *Aire*. Neither concentrations of LH nor FSH in the serum were significantly different between WT and *Aire*^{-/-} animals (LH: WT, 0.07 ± 0.03 ng/mL, *n* = 5; *Aire*^{-/-}, 0.19 ± 0.08 ng/mL, *n* = 13; *P* = 0.37) (FSH: WT, 17.75 ± 1.32 ng/mL; *Aire*^{-/-}, 22.05 ± 2.56 ng/mL; *P* = 0.33). However, serum testosterone was reduced by >50% in all males, including the two males that produced small litters (Figure 6A).

To examine sperm abundance and motility, epididymides were dissected and weighed, and spermatozoa from

Figure 11 Autoimmunity against the prostate gland of *Aire*^{-/-} mice. **A** and **B**: Representative sections of prostate from wild-type (WT) or *Aire*^{-/-} mice were stained with hematoxylin and eosin (**A**) or antibody against CD3 (**B**). Boxed area in **A** is shown at higher magnification to the right. **C**: Prostate lysate from a *Rag2*^{-/-} mouse was probed with sera from WT and *Aire*^{-/-} mice. Each lane represents an individual mouse; ages in weeks are indicated at the top, and numbers on right represent the molecular weight marker (kDa). *n* = 7 WT mice (**A** and **B**); *n* = 20 *Aire*^{-/-} mice (**A** and **B**). Scale bars: 100 μ m (**A**); 50 μ m (**B**).

the caudal region were obtained by the swim-up method. Caudal epididymal weight of *Aire*^{-/-} mice was not significantly different from that of controls (WT: 8.18 ± 0.27 mg, *n* = 8; *Aire*^{-/-}: 9.66 ± 0.60 mg, *n* = 9; *P* = 0.11), and sperm recovery was similar between WT and *Aire*^{-/-} mice (Figure 6B). In a separate group of mice, neither DSP nor sperm production efficiency differed significantly between WT and *Aire*^{-/-} males (Figure 6, C and D). Computer-assisted sperm analysis in two of four *Aire*^{-/-} males examined revealed total and progressive sperm motility was comparable to those of WT controls (data not shown). In the remaining two *Aire*^{-/-} males, however, epididymal sperm were undetectable. One of these males (number 851) (Figure 6, C and D) also showed dramatically reduced DSP and sperm production

efficiency, whereas the other (number 835) exhibited DSP and sperm production efficiency comparable to the WT controls and other *Aire*^{-/-} males; both mice had normal testis weight. A third male (number 1297), which had reduced testis weight, had low DSP but normal sperm production efficiency.

In vitro fertilization was performed to directly examine sperm function in *Aire*^{-/-}. Fertilization with WT sperm produced two-cell and blastocyst stage embryos with a success rate of 54% and 40% of total oocytes, respectively (Figure 6E). However, sperm from *Aire*^{-/-} males had only a 9% success rate in producing two-cell embryos, none of which progressed to the blastocyst stage.

Identification of Potential Testis Target Autoantigens

Bioinformatic analysis of an existing data set⁴⁰ was used to identify *Aire*-regulated testis-specific genes that, if unexpressed in *Aire*^{-/-} thymus, might contribute to autoimmune disease targeting this organ. To this end, the over representation of *Aire* target genes was evaluated in different sets of tissue-specific genes across diverse tissues. First, 47 genome-wide RNA-sequencing data sets were integrated from two previous studies^{38,43} that cover comprehensive gene expression of multiple tissues, including testis. Sets of tissue-specific genes were identified for each tissue (Supplemental Figure S2) on the basis of differential gene expression patterns across these tissues (Supplemental Figure S1). On average, there were 680 tissue-specific genes per tissue. Interestingly, testis expressed the highest number of tissue-specific genes of any tissue (approximately 2500 testis-specific genes). Gene Ontology enrichment analysis revealed cellular functions associated with spermatogenesis, meiosis, cilium morphogenesis, axoneme assembly, inner dynein arm assembly, Piwi-interacting RNA biogenesis, and fertilization (Supplemental Figure S3).

Aire target genes were highly enriched in gene sets specifically expressed in the testis, thymus, and other tissues (Figure 7). For testis, >200 testis-specific genes targeted by *Aire* were identified for both up-regulation and down-regulation (*P* < 10^{-9}) (Supplemental Table S1).

Widespread Autoimmunity in the Reproductive Tract of *Aire*^{-/-} Males

In contrast to the testes, lymphocytic infiltration occurred in epididymides of 15 of 22 *Aire*^{-/-} males, as opposed to those of WT males examined (Figure 8, A and C). This held true even if epididymal gross morphology appeared normal, and if epididymal sperm were present. A complete absence of epididymal sperm was observed in six mice, all of which displayed abnormally interstitial morphology, characterized by increased intratubular space, fibrosis, and loss of epithelial integrity. The presence of T cells was confirmed by probing epididymal sections with anti-CD3 antibody. T cells were observed within caput, corpus, and cauda of the

epididymis, sometimes in aggregates or within ductal epithelium (Figure 8, B and D). Binding of serum autoantibodies to caput (data not shown) and cauda epididymal sections of *Rag2*^{-/-} mice consistently revealed reactivity to apical epithelium and connective tissue in all mice examined, as well as to caudal sperm in three of four mice (Figure 9A). Western blot analysis of isolated caudal epididymal protein revealed strong autoantigen reactivity of *Aire*^{-/-} serum to multiple proteins as early as 8 weeks of age, and in all mice examined by 21 weeks (Figure 9B). Particularly prominent were autoantigens of 20, 25, 37, and approximately 45 kDa. Finally, direct immunofluorescence detected prominent antibody deposition within the epithelium, basement membrane, and connective tissue of epididymal tubules (Figure 9C). As in testis sections, sperm were not directly bound.

Seminal vesicles were also examined in WT and *Aire*^{-/-} mice, and although gross morphology of these glands was unaltered, a mild lymphocytic infiltrate, including CD3⁺ T cells, within the loose connective tissue below the mucosal epithelium was observed in 16 of 19 *Aire*^{-/-} mice (84%) (Figure 10, A and B). Autoantibodies against the seminal vesicles were generated, although these occurred in a minority of mice only after 6 months of age (Figure 10C). Finally, the presence of pronounced prostatitis was confirmed in 17 of 19 (89%) of *Aire*^{-/-} mice and anti-prostate autoantibody formation (Figure 11), as reported previously.^{29,44}

Aire mRNA Is Expressed in the Testis

To determine whether *Aire* is expressed in male reproductive tissues, we analyzed transcript and protein expression using RT-qPCR, immunofluorescence, and an *Aire-Cre-mTmG* reporter system (Figure 12). RT-qPCR analysis for *Aire* mRNA expression showed low but detectable expression in the testis at 3 and 10 to 12 weeks of age, whereas expression was not detected in the seminal vesicles or the prostate gland (Figure 12A). Our *mTmG* reporter recapitulated the expected pattern of *Aire* in mTECs of the thymus (Figure 12, B and C), and although no expression was observed in the seminal vesicle, reporter expression was observed in the prostate gland (Figure 12, D and E). The reporter system also showed that the *Aire* gene was expressed in the testis, specifically in the seminiferous epithelium, at 3 weeks of age, and spermatids were present in 9-week-old males (Figure 12, F and G). *Aire* protein expression in the testis was studied using immunofluorescence. Although *Aire* expression was readily detected in the thymus (Figure 12H), it was not seen in the

testis, either at 3 or 12 weeks of age (data not shown) (Figure 12I). *Aire* protein expression was not detected in the prostate gland of mice at these ages.

Discussion

Mutations of the *AIRE* genes in both humans and mice impede the establishment of central immune tolerance and produce multiorgan autoimmune disease, characterized by the presence of autoreactive T cells and antibodies.^{15,17,45} Although differences exist between the organs of *APS-1* patients and *Aire*^{-/-} mice, a shared pathology is premature gonadal insufficiency.^{17,25–27,45} One cause of early reproductive senescence in female mice on the BALB/c genetic background is an age-dependent, autoimmune-associated loss of ovarian follicular reserves and failed embryonic development.^{33,46} However, information on fertility and reproductive immune targets in male *Aire*-deficient mice has been scattered and incomplete. This study showed that most male *Aire*^{-/-} mice on the Balb/cJ genetic background were infertile; the few mice that did produce offspring had small litter sizes. *Aire*^{-/-} mice also produced low levels of testosterone and developed autoimmune disease against many components of the male reproductive tract. Furthermore, sperm produced by *Aire*^{-/-} mice had severely diminished fertilizing potential, and a subset of males had oligospermia with an apparent disruption of the blood-testis barrier.

In men with APS-1, gonadal insufficiency occurs in approximately 12% to 14% of cases,¹⁴ with most being diagnosed with primary hypogonadism and associated low testosterone levels; hypogonadotropism is not usually found in these patients. Male *Aire*^{-/-} mice share these changes. Interestingly, more than half of *Aire*^{-/-} mice also failed to mate over a 3-week period. Testosterone levels were roughly one-third of those of WT mice. Produced by Leydig cells, testosterone controls sexual behavior in mice and in vertebrates⁴⁷; thus, reduced testosterone in *Aire*^{-/-} mice may contribute to their lack of copulation, as evidenced by infrequent detection of copulatory plugs.

Although most animals examined in this study exhibited normal gross and histologic testicular morphology, a subset of mice had severe autoimmune orchitis with reduced testis size, depletion of germ cells, and reduction of DSP. This was associated with serum autoantibody formation and infiltration of T cells and macrophages, which may contribute to the destruction of testosterone-producing Leydig cells. More importantly, mature spermatozoa

Figure 12 Expression analysis of *Aire* in the male reproductive tract. **A:** Quantitative RT-PCR analysis of *Aire* in indicated organs of 3-week-old and 10- to 12-week-old males. **B:** Schematic representation of generation of *Aire*-Cre reporter mice. *Aire*^{Cre/WT} males were bred with female mTmG mice, resulting in 50% of the offspring *Aire*^{Cre/WT};mTmG. Males of this genotype were sacrificed at 3 or 9 weeks of age, and thymus (**C**) and reproductive tract tissues [seminal vesicle (**D**), prostate gland (**E**), testis (**F**, 3-week-old male; **G**, 9-week-old male)] were dissected and analyzed by epifluorescence microscopy. **H** and **I:** Immunofluorescence imaging of *Aire* in thymus (**H**) and testis (**I**) of a 3-week-old male. Scale bars: 2000 μm (**C**); 50 μm (**D**, **H**, and **I**); 200 μm (**E**); 100 μm (**F** and **G**).

contain numerous specific proteins⁸ that are potentially immunogenic in both men and women. Anti-sperm antibodies can form following trauma, inflammation, or vasectomy,⁴⁸ and are present in significantly higher proportion in infertile men than in fertile men.^{49,50} These antibodies are clinically important when >50% of the spermatozoa are coated with antibodies that can block sperm penetration and decrease *in vitro* fertilization rates.^{51,52} Hubert et al²⁶ speculated that anti-sperm autoantibodies are causative for decreased fertility in *Aire*-deficient males on the B6 background. Intriguingly, our bioinformatics analysis revealed a plethora of testis-specific genes regulated by *Aire* in the thymus, and thus identified potential autoimmune targets. Although the blood-testes barrier plays an important role in sequestering germ cell from insult by the immune system,⁵³ no endogenous antibodies were seen in seminiferous tubules of *Aire*^{-/-} mice, suggesting that anti-sperm antibodies in these mice do not reach sperm in the testis *in vivo*. The blood-testes barrier may have remained intact, at least before fulminant inflammation. It seems likely that *Aire* may function through its role in regulating deletion of self-reactive T cells, in turn regulating antibody production and/or shaping of regulatory T cells. Future studies will dissect the causative relationships between anti-sperm T cells, antibodies, and infertility.

The study showed significantly lower *in vitro* fertilization success rates, with only 9% of wild-type oocytes developing to the two-cell stage, and none reaching the blastocyst stage, after being incubated with epididymal sperm from BALB/c *Aire*-deficient mice. It is possible that the observed orchido-epididymitis impacts the health and quality of the developing sperm, causing reduced fertilization potential. Although both reduced testosterone and local/systemic testicular inflammation can contribute to germ cell loss and infertility,⁵⁴ only approximately 15% of the *Aire*^{-/-} male mice had evidence of acute testicular inflammation. On the other hand, 68% of the animals had ongoing inflammation and/or fibrosis that suggested a previous inflammatory event. There was considerable autoantibody binding to basal epithelium of epididymal tubules. Epididymitis is the most common male reproductive tract inflammatory disease, impacting >600,000 males annually, and can lead to secondary involvement of the testis.^{55,56} Secretory products of epididymal epithelium contribute to completion of sperm development, enhancement of motility, and the ability to bind the zona pellucida.^{57,58} We hypothesize that the epididymis is the initial target of the male reproductive tract in *Aire*-deficient mice, and that abundant antibody deposition within the epididymis alters the ability of tubular epithelium to transport and secrete the requisite seminal plasma proteins for complete spermatozoa development, thereby impairing their fertilization potential.

Chronic prostatitis and chronic pelvic pain syndrome in men is a common but poorly understood condition. Patients present urologic symptoms, nonspecific discomfort in the pelvic region, and sexual dysfunction.⁵⁹ Despite

initial speculation that these disorders had a bacterial etiology, it is now believed that most chronic prostatitis and chronic pelvic pain syndrome cases have noninfectious origins.^{56,60} The presence of prostatic immune aggregates in chronic prostatitis and chronic pelvic pain syndrome is common,^{61,62} and some patients can generate a recall T-cell response when challenged with seminal plasma,⁶³ both of which suggest a potential autoimmune component to chronic prostatitis and chronic pelvic pain syndrome. *Aire*-deficient mice on a mixed (129/Sv × C57BL/6) F₂ genetic background develop spontaneous immunity to the prostate autoantigen seminal vesicle secretory protein 2,²⁹ resulting in moderate to severe prostatitis in >70% of the mice. In the current study, this result was confirmed by nearly 90% of the animals on a congenic BALB/c background developing severe prostate lymphocytic infiltration and autoantibody generation. Background genetics has a significant influence on the severity of disease and the range of autoantigens targeted in mouse models of *Aire* deficiency.²⁸ However, some target organs, such as the prostate, are consistent across strains. Interestingly, the dominant prostate antigens in *Aire*^{-/-} BALB/c mice are not seminal vesicle secretory protein 2, but instead a higher-molecular-weight protein, identification of which is currently underway. Nevertheless, a deficiency in *Aire* is a good model for the study of chronic autoimmune prostatitis.

These studies of expression of *Aire* in the male reproductive tract raise the possibility that *Aire* in developing sperm contributes to fertility. RT-qPCR and reporter gene analyses confirm that the *Aire* gene is expressed in the seminiferous epithelium during the first wave of spermatogenesis. Reporter expression was observed in developing sperm, possibly pachytene spermatocytes, and early round spermatids.⁶⁴ Interestingly, reporter expression progressed to elongated spermatids in the 9-week-old mouse but did not reappear in earlier stages of spermatogenesis at this age, suggesting that *Aire* expression is transient. This result is in general agreement with that of Schaller et al,⁶⁵ who reported *Aire* expression in spermatogonia of 3-week-old, but not older, mice. Unlike that study, however, this study did not have expression in the spermatogonia, which lie along the basement membrane, suggesting that *Aire* is transcribed after this stage. Because the initial wave of spermatogenesis is essential for proper development of fertility in mice, the expression of *Aire* in these cells may contribute directly to male fertility, independently of the immune system.

Aire reporter gene expression was also observed in the prostate gland. *Aire* is regulated by androgen receptor, which is expressed by the developing prostate gland.^{66,67} In contrast, mRNA in this tissue was undetectable. Collectively, these results raise the possibility that transient expression of *Aire* occurs in the prostate during embryogenesis and/or early postnatal life through regulation by the androgen receptor.

In summary, we have found that targeted deletion of *Aire* in male mice results in fertility problems similar to those afflicting men with APS-1. Infertility in these animals appears to be multifactorial. Lack of mating behavior, possibly due to low testosterone, may contribute to infertility, as could defects in spermatogenesis. However, these symptoms affect only 50% and 20% of animals, respectively. On the other hand, sperms themselves may be incapable of fertilization, as only a minor proportion of donors were able to produce two-cell embryos. These problems may be indicative of epididymal defects, as most animals had inflammatory infiltration into the epididymis, as well as anti-epididymal autoantibodies. The correlation between impaired central immune tolerance and fertility has potential implications for not only male APS-1 patients but may also provide important insights into both male autoimmune and unexplained cases of infertility.

Author Contributions

B.D.W., B.K.P., P.S.K., M.K.N., G.B., S.H.A., and M.G.P. designed experiments and interpreted data; B.D.W., M.K.N., G.S., S.H.A., and K.S.B. performed experiments; H.W. and J.W. designed and performed bioinformatic analyses; B.D.W., S.H.A., and M.G.P. wrote the manuscript; all authors edited the manuscript.

Supplemental Data

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