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Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein

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Humans expressing a defective form of the transcription factor AIRE (autoimmune regulator) develop multiorgan autoimmune disease. We used *aire*-deficient mice to test the hypothesis that this transcription factor regulates autoimmunity by promoting the ectopic expression of peripheral tissue–restricted antigens in medullary epithelial cells of the thymus. This hypothesis proved correct. The mutant animals exhibited a defined profile of autoimmune diseases that depended on the absence of aire in stromal cells of the thymus. Aire-deficient thymic medullary epithelial cells showed a specific reduction in ectopic transcription of genes encoding peripheral antigens. These findings highlight the importance of thymically imposed "central" tolerance in controlling autoimmunity.

A problem that has intrigued immunologists for decades is how animals achieve immunological tolerance to autoantigens (*1*). For example, T cells are generated in the thymus, and because their antigen-specific receptors are encoded by genes assembled through random somatic DNA rearrangement, the emergent repertoire of receptors inevitably includes specificities capable of reacting to self constituents. To avoid the potentially pathological state of autoimmunity, it is necessary to purge these self-reactive cells from the repertoire, either by removal or silencing. Some are removed in the thymus soon after generation, but this raises the question of how thymocytes that are reactive to proteins expressed only in nonthymic parenchymal tissues can be identified and dealt with. A commonly held notion involves a dichotomy of "central" and "peripheral" mechanisms: Tolerance to ubiquitously expressed or blood-borne antigens is achieved in the thymus, whereas tolerance to tissue-restricted antigens is secured by means of diverse extrathymic processes.

Surprisingly, RNA transcripts encoding a multiplicity of proteins previously considered to be synthesized only in particular peripheral tissues can be detected in the thymus (*2, 3*), specifically in very rare epithelial cells in the medulla (*4, 5*). Examples include transcripts encoding transcription factors, structural proteins, membrane proteins, hormones, and secreted proteins. Thymic medullary epithelial cells (MECs) have been increasingly implicated in the clonal deletion or inactivation of semimature self-reactive thymocytes (*6, 7*), fueling interest in the precise function of these ectopically expressed transcripts. Several transgenic (*4, 8–17*) and nontransgenic (*18, 19*) mouse systems have revealed a direct link between ectopic synthesis of a designated protein in MECs and the absence of peripheral lymphocyte reactivity to that protein. Many of the ectopically expressed antigens (insulin, thyroglobulin, myelin basic protein, and retinal S-antigen) are associated with organ-specific autoimmune diseases (type 1 diabetes, thyroiditis, multiple sclerosis, and uveitis, respectively). In addition, there are some very suggestive correlations between antigen expression levels in the thymus and disease susceptibility in humans (*20, 21*) and rodents (*22*). We sought to determine what drives ectopic synthesis of peripheral tissue–restricted proteins in MECs, and what impact this expression has on an animal's state of immunological self tolerance.

Following clues from the human system, we anticipated that mice lacking the *aire* gene would prove key to addressing these issues. Autoimmune polyendocrinopathy–candidia-

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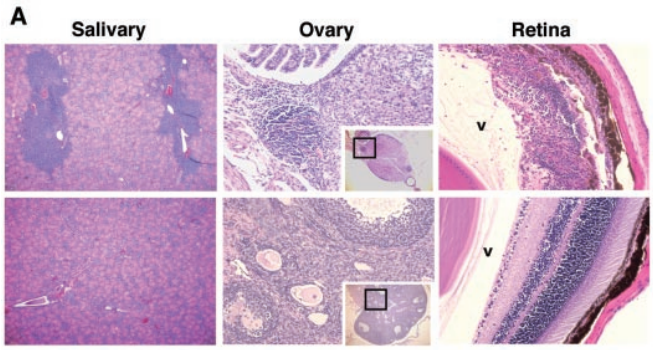
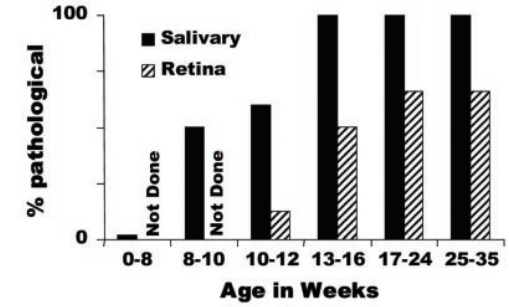
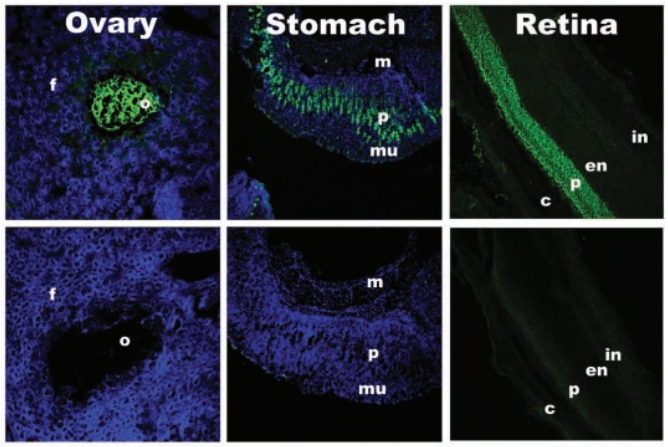
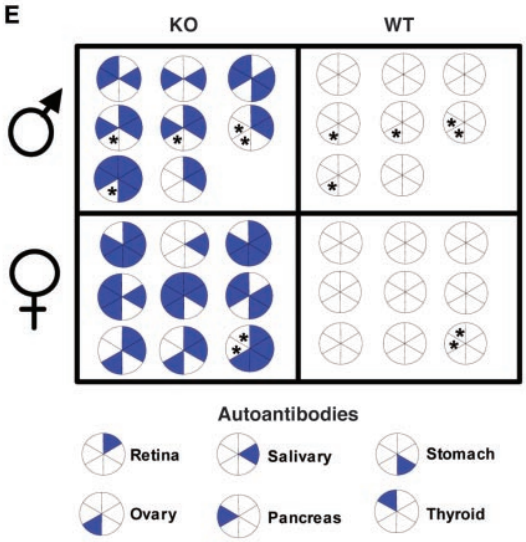


B

Sex	Age	Salivary	Reproduc.	Thyroid	Retina	Liver	Stomach
F	3	-	-	+	ND	-	-
M	4	-	-	-	ND	-	-
M	4	-	-	-	ND	-	-
F	9	+	-	-	ND	-	-
F	9	+	-	ND	ND	ND	ND
M	9	-	-	ND	-	ND	ND
M	10	tr	-	ND	+	ND	ND
M	15	+	-	-	ND	-	-
F	15	+	+	+	+	tr	-
F	15	+	-	-	-	tr	-
F	22	+	-	NA	+	tr	-
M	30	+	+	NA	-	+	+
F	31	+	tr	-	+*	+	+
M	35	+	+	NA	+	+	-







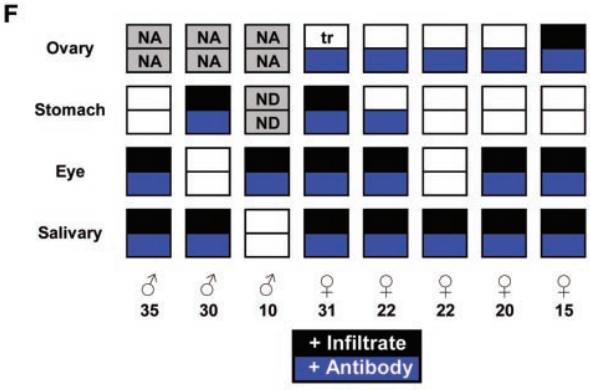


Fig. 1. Aire-deficient mice develop autoimmunity. (**A**) Tissue sections from aire-deficient mice from salivary gland (magnification ×50, 35-week-old males), ovary (×200, inset ×50 with area of ×200 view highlighted, 15-week-old females), and retina (×200, 31-week-old females; v, vitreous) from aire-deficient (i.e., knockout; top row) and wild-type (bottom row) mice stained with hematoxylin and eosin (H&E). Each knockout section shows representative lymphocytic infiltrates. (**B**) Table summarizes presence (+) or absence (–) of lymphocytic infiltrates observed in knockout mice of various ages and both sexes for the indicated organs. "Reproduc." refers to ovaries in female mice and to the coagulating glands in male mice. For the retina, many mice had retinal degeneration and were scored +; in addition, one mouse had retinal degeneration with immune infiltration and is scored +*. NA, not available; ND, not done; tr, trace infiltrate. Age- and sex-matched wild-type mice were also examined, and no infiltrate was seen in any mouse in any of the indicated organs (n = 14). (**C**) Salivary gland infiltrates and retinal degeneration develop in a time-dependent manner. Shown are percentages of mice with salivary gland infiltrates or retinal degeneration killed at the given age ranges; n = 21 for the salivary gland and n = 16 for the retina. (**D**) Aire-deficient mice have autoantibodies to multiple organs. Shown is representative staining by indirect immunofluorescence on tissue sections of ovary (magnification ×250), stomach (×100), and retina (×250) for serum from individual knockout (top row) or age- and sex-matched wild-type (bottom row) mice. Green indicates serum staining; blue indicates staining with the nuclear marker 4′,6′-diamidino-2-phenylindole (DAPI) (not done on retina sections). For the ovary, knockout serum reacted against the cytoplasm of the oocyte (marked o; f, follicle). For the stomach, knockout serum stained the parietal cell layer (marked p; mu, mucinous layer; m, muscular layer). The retinal staining revealed knockout serum that reacted against the outer retinal layer containing the epithelium and rods and cones (marked p; c, choroid; en, external nuclear layer; in, inner nuclear layer). (**E**) Summary of autoantibodies in aire-deficient mice. Serum from age- and sex-matched mice (9 to 35 weeks old) was collected and tested for the presence of autoantibodies to the indicated organs. Each circle represents an individual mouse, with the corresponding autoantibody indicated by the key below the chart; *, not done. (**F**) Summary of correlation of autoantibodies to immune infiltrates for ovary, salivary gland, retina, and stomach in seven knockout mice. For each mouse of the indicated sex and age (in weeks), detection of an infiltrate or an autoantibody is indicated. NA, not available; ND, not done; tr, trace infiltrate.

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sis–ectodermal dystrophy (APECED), also known as autoimmune polyendocrine syndrome–type 1 (APS-1), is a polyglandular disorder that classically manifests as spontaneous autoimmunity against the parathyroid and/or adrenal glands, and/or by a mucocutaneous candidiasis infection [reviewed in (*23, 24*)]. Other common ailments include autoimmune forms of premature ovarian failure, hepatitis, anemia, diabetes, alopecia, and vitiligo. Disease is inherited in an autosomal recessive manner as a result of loss of function of a single susceptibility gene, which encodes the AIRE protein. AIRE has shown DNA binding activity (*25*) and transcriptional transactivation potential (*26, 27*) in assays in vitro. This protein and its murine homolog, aire, have a pattern of expression that suggests a potential role in shaping the T cell repertoire: By far the highest amounts are found in the thymus, specifically in MECs (*4, 28, 29*). It seemed reasonable to hypothesize that APECED patients develop multiorgan autoimmunity because a defect in AIRE prevents or modifies ectopic transcription of genes encoding peripheral tissue–restricted antigens in thymic MECs. A recent report that aire-deficient mice also exhibit autoimmune manifestations is consistent with this proposal, although that study concluded that the cause was a problem with peripheral lymphocyte homeostasis (*30*). Here, we investigated an independently generated line of aire-deficient mice in an effort to learn whether the spectrum of autoimmunity manifested by these mice is consistent with a broad defect in central tolerance induction, and whether this results from an alteration in ectopic expression of tissue-restricted antigens by thymic MECs.

We engineered a line of mice carrying a defective *aire* gene by means of lox/cre-mediated recombination in embryonic stem (ES) cells (fig. S1). These mice carry in homozygous state a mutant allele bearing a deletion of exon 2 and portions of the upstream and downstream introns (fig. S1A). Mature *aire* gene transcripts could be detected at near-normal levels in the thymus of mutant mice, but these were smaller than in the wild type, as expected given the absence of exon 2 (fig. S1B). Sequence analysis confirmed that these transcripts could not give rise to functional protein because a frame shift provoked by the abnormal juxtaposition of exons 1 and 3 resulted in premature truncation shortly after exon 1 (fig. S1C). The *aire* gene mutation was generated in ES cells derived from mice of the Sv/129 strain, and mice used in the experiments described here were from F_2 or F_3 backcrosses onto the B6 genetic background.

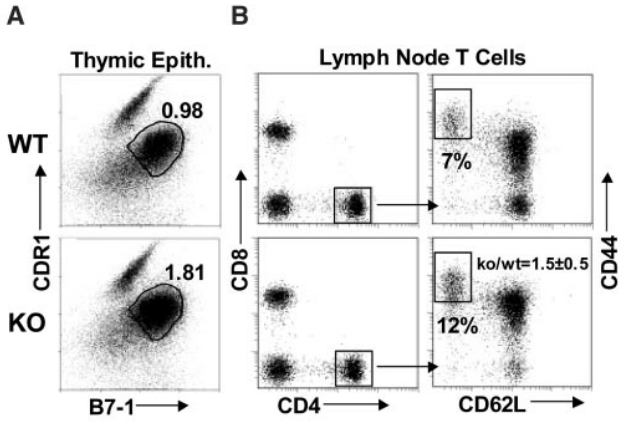

Fig. 2. Flow cytometric analysis of lymphoid organs in aire-deficient mice. (**A**) Increased number of thymic MECs in knockout mice. Pooled thymic stromal cells from five knockout (KO) or wild-type (WT) mice were stained with anti-CD45, CDR1, G8.8, and anti-B7-1. The plots contain only the $CD45^{-}G8.8^{+}$ cells. Cortical epithelial cells are $CDR1^{hi}B7\text{-}1^{lo}$, and MECs (circled) are $CDR1^{int}B7\text{-}1^{hi}$. Numbers ($\times 10^5$) of cells in each gate are shown from one of four representative experiments. (**B**) Increased activation/memory markers in aire-deficient T cells. Cells from peripheral lymph nodes of individual mice were isolated and stained. Shown are representative plots for a wild-type and a knockout mouse with $CD4^{+}$ cells gated on the left and activation markers CD44 and CD62 ligand (CD62L) on the right. The percentages of $CD4^{+}$ cells that are $CD44^{hi}CD62L^{lo}$ for a representative knockout and wild-type mouse are indicated. The ratio of the knockout $CD44^{hi}CD62L^{lo}$ percentage versus the wild-type $CD44^{hi}CD62L^{lo}$ percentage for $CD4^{+}$ cells from 10 knockout and 9 wild-type mice 3 to 15 weeks of age is also indicated. A similar increase in $CD44^{hi}CD62L^{lo}$ cells was also seen for $CD8^{+}$ cells (*32*).

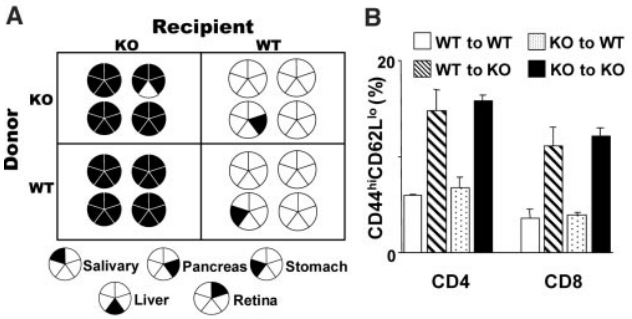

Fig. 3. Absence of aire in stromal cells leads to autoimmunity. (**A**) Bone marrow from knockout or wild-type mice was transferred into lethally irradiated recipient knockout or wild-type mice in a classic crisscross fashion, yielding four experimental groups. Organs were harvested 2 months after reconstitution and analyzed for autoimmunity. The circles represent individual mice and summarize the pattern of immune infiltrates from 16 mice (four from each group) in the indicated organs (see fig. S8 for representative H&E stains). Autoantibodies were also detected for stomach, salivary gland, pancreas, liver, and retina; these followed an identical pattern to the infiltrate data and are not shown separately. (**B**) Analysis of activation markers on $CD4^{+}$ and $CD8^{+}$ T cells from bone marrow chimeras. Inguinal lymph nodes were harvested and analyzed by flow cytometry for CD4, CD8, CD44, and CD62L. Data represent four mice in each group in two experiments. T cells from spleens, cervical lymph nodes, mesenteric lymph nodes, and pancreatic lymph nodes exhibit similar patterns (*32*).

Aire prevents autoimmunity. We first tested whether the absence of aire in these mice leads to broad defects in tolerance induction. Lymphocyte infiltrates were observed in several organs, usually confined to particular structures rather than being distributed indiscriminately throughout the organ. For example, lymphocytes were seen in the salivary gland only in the perivascular regions, in the ovary only in the follicles, and in the eye only in the retina (Fig. 1A). The infiltrates showed a pronounced age dependence by two criteria: (i) The number of organs targeted increased in older aire-deficient mice, ranging from none or one at 3 to 4 weeks of age to at least four or five after 30 weeks (Fig. 1B). (ii) For any particular organ, the fraction of mutant mice with autoimmune manifestations increased with age. For example, salivary gland infiltrates were rare in mice less than 8 weeks of age but were constant after 13 weeks (Fig. 1C). Autoantibodies had the same kind of target distribution. Multiple organs were targeted, but only at specific substructures within each (e.g., oocytes in the ovary, parietal cells in the stomach, the outer layer of the retina in the eye). All aire-deficient mice had serum autoantibodies after 3 to 6 months of age, most with reactivity to several organs, as detected by staining of tissues from wild-type mice (Fig. 1D). In the majority of aire-deficient mice, there was a good correlation between organs targeted by infiltrates and those targeted by autoantibodies (Fig. 1F). In short, the phenotype of these aire-deficient mice is certainly compatible with a broad defect in tolerance induction, but one characterized by highly selective, rather than indiscriminate, autoimmune attack.

We next sought to determine whether the

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autoimmunity exhibited by aire-deficient mice was reflected in other immune system abnormalities. Most of the features examined histologically, cytofluorimetrically, or via functional assays appeared within the normal range (*31*) (figs. S2 to S7), in general agreement with previous findings (*30*). These included thymocyte numbers, CD4/CD8 subset distributions, and differentiation marker profiles; bone marrow cell numbers and immunoglobulin M (IgM)/B220 profiles; spleen and lymph node cell numbers, IgM/TCR (T cell receptor)–αβ, IgM/IgD, and CD4/CD8 profiles and $CD4^+CD25^+$ regulatory T cell compartments; dendritic cell numbers and subsets; T cell cytokine production after anti-CD3/CD28 stimulation; in vitro presentation of antigens or peptides; stimulatory and response capacity in mixed lymphocyte reactions; and thymus, spleen, and lymph node histology. Two features of the immune system in mutant mice were abnormal, however. There was an average doubling of the number of MECs (Fig. 2A), although expression of major histocompatibility complex (MHC) class II and costimulatory molecules were normal (*32*) and there was a near-doubling in the frequency of activated/memory $CD44^{hi}CD62L^{lo}$ T cells in both the $CD4^+$ and $CD8^+$ compartments of the peripheral lymphoid organs of most mice (Fig. 2B). This latter observation is consistent with the possibility that aire somehow functions to reduce the self reactivity of peripheral T cells.

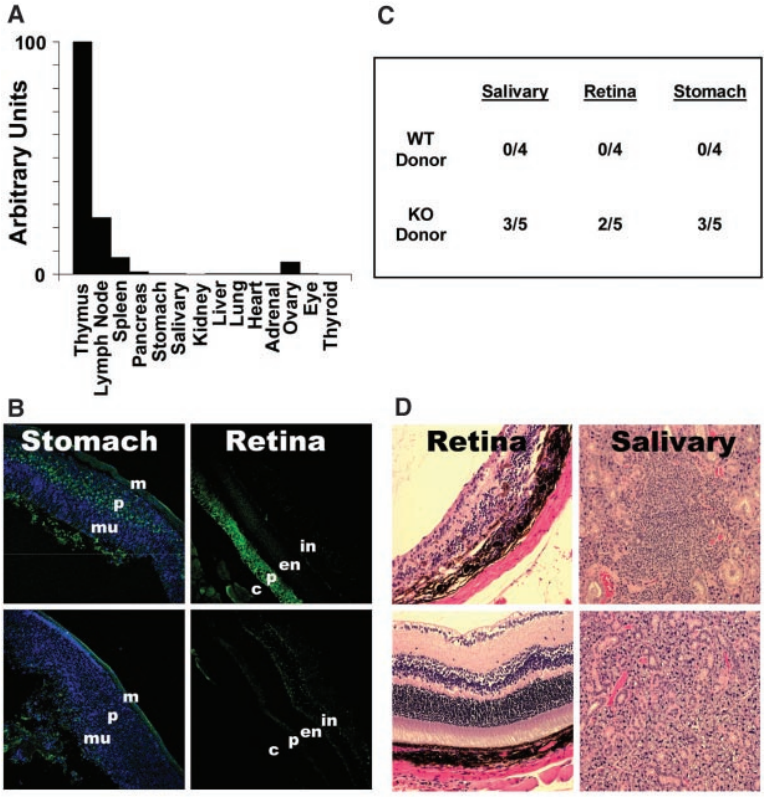

Fig. 4. The thymus is critical for aire-associated autoimmunity. (**A**) Aire is expressed predominantly in the thymus. Shown is relative expression of aire by quantitative real-time PCR using Taqman primers and probes on cDNA prepared from various whole organs from a 6-week-old B6 mouse. Normalization of cDNA content was done on cyclophilin; numbers represent a ratio of relative aire expression to cyclophilin expression for each tissue. For tissues other than thymus, lymph node, spleen, and ovary, aire transcripts could not be detected. Given the normalization of cDNA content by cyclophilin, the amount of aire in these tissues is at least two orders of magnitude less than the level seen in the thymus. Data shown are a representative experiment of several independent experiments. (**B**) Transplantation of aire-deficient thymi leads to autoantibody production. Nude mice were transplanted with either aire-deficient ($n = 3$) or wild-type thymi ($n = 6$). Six weeks after transplantation, serum was collected and tested for autoantibodies against stomach (×100) and retina (×250) by indirect immunofluorescence for recipients of aire-deficient (top panels) and wild-type control (bottom panels) thymi. Shown is representative staining from individual mice. Green indicates specific serum staining; blue (only on stomach sections) indicates staining with the nuclear marker DAPI. For the stomach, serum (3/3 knockout, 0/6 wild type) stained the parietal cell layer (p) of the stomach mucosa (mu, mucinous layer; m, muscular layer). For the retina, serum (2/2 knockout, 0/4 wild type) reacted against the outer layer containing the epithelium and rods and cones (marked p; c, choroid; en, external nuclear layer; in, inner nuclear layer). (**C**) Mature aire knockout lymphocytes can transfer immune infiltrates. Table summarizes immune infiltrates observed (12 weeks after transfer) in the salivary gland, retina, or stomach for Rag-deficient recipients injected with aire knockout lymphocytes or wild-type lymphocytes. Numbers represent individual mice for each group. (**D**) Representative immune infiltrates in tissue sections stained with H&E from Rag-deficient mice injected with lymphocytes from aire knockout mice (top panels) or wild-type control mice (bottom panels) for retina (×200) and salivary gland (×100).

Requirement for aire expression in the thymic stroma. To establish whether the autoimmune manifestations in aire-deficient mice are attributable to its absence from radioresistant or hematopoietic cells, we constructed a set of radiation bone marrow chimeras, which produced aire in radioresistant cells only, in hematopoietic cells only, in neither cell type, or in both. Signs of autoimmunity were detected only in those chimeras that lacked aire in the radioresistant cells. Lymphocytic infiltrates were found in all irradiated aire-deficient recipients, whether the donors of bone marrow cell precursors expressed aire or not. The invasiveness of the infiltrates was strikingly enhanced in these chimeras vis-à-vis unmanipulated mutant mice according to multiple criteria: the fraction of young mice showing infiltrates, number of organs infiltrated, and extent of the infiltrate (Fig. 3A) (fig. S8). Likewise, irradiated aire-deficient recipients of both wild-type and mutant bone marrow cell precursors exhibited serum autoantibodies, and these were more prevalent and had a wider target range than was found with unmanipulated mutant mice (Fig. 3A). Again, the profiles of organs targeted by infiltrates and by autoantibodies were closely correlated (Fig. 3A). Finally, the abnormal accumulation of activated/memory $CD44^{hi}CD62^{lo}$ cells, both $CD4^+$ and $CD8^+$, also correlated with the absence of aire in radioresistant cells (Fig. 3B). Thus, the autoimmune manifestations in aire-deficient mice reflect this protein's function in radioresistant cells.

The next issue to resolve was whether aire exerts its influence on autoimmunity within the thymus or the periphery. Although aire and its human equivalent are predominantly synthesized in the thymus, expression in a number of peripheral tissues has also been reported, including several of those targeted by lymphocytic infiltrates or autoantibodies in aire-deficient individuals (*33, 34*). However, expression in peripheral parenchymal tissues has been controversial (*35, 36*), primarily because of its weakness and irreproducibility (*35, 36*). We carefully examined *aire* gene expression in normal mice by quantitative real-time polymerase chain reaction (PCR) (Fig. 4A). Transcripts were by far the most abundant in the thymus. Although transcripts were also detected in peripheral lymphoid organs (at significantly lower levels), they were essentially undetectable in the parenchymal tissues examined other than the ovary. These results imply that target tissues need not synthesize significant levels of aire to avoid

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autoimmune attack and indicate the importance of aire expression in lymphoid tissues.

To distinguish the influence of the thymus from that of peripheral lymphoid organs, and to further rule out any role for peripheral parenchymal tissues, we performed a thymus graft experiment. A thymus from either an aire-deficient mouse or a wild-type littermate was depleted of hematopoietic cells by incubation in 2-deoxyguanosine (*31*) and was then transplanted into an athymic $B6^{nu/nu}$ recipient. Six weeks later, the generation of a peripheral T cell compartment was confirmed, and serum autoantibodies were assayed on stomach and eye sections from normal mice. Autoantibodies were found in sera from each of three mice hosting an aire-deficient thymus but in none of six hosts of a wild-type thymus, although the T cell compartments were reconstituted to the same level in the two cases. The pattern of autoantibody staining was the same as that seen with sera from unmanipulated aire-deficient mice (compare Figs. 4B and 1B). We thus concluded that aire must be expressed in the radioresistant stromal cells of the thymus in order to control autoimmunity in the periphery.

Influence of aire on T cell education. We also established that the autoimmune disease that develops in aire-deficient mice is lymphocyte-autonomous. Mature lymphocytes were pooled from the spleen and lymph nodes of aire-deficient mice or wild-type littermates and were then transferred into alymphoid $Rag^{o/o}$ recipients. After 12 weeks, diverse organs were assayed histologically. Lymphocytic infiltrates, similar to those observed in unmanipulated mice lacking aire, were found in the salivary gland, eye, and stomach of the mice that received aire-deficient, but not wild-type, lymphocytes (Fig. 4, C and D). These results confirm that aire expression in peripheral parenchymal tissues is not the factor controlling autoimmune attack.

Aire regulates thymic gene expression. Given the association between aire deficiency and autoimmunity, and the predominant expression of this transcription factor (*25–27*) in thymic MECs (*4, 5*), we hypothesized that aire controls autoimmunity by regulating the ectopic expression of peripheral tissue–restricted antigens in the medulla of the thymus. MECs were present (indeed, they were overrepresented) in aire-deficient mice and displayed normal levels of molecules associated with antigen presentation (fig. S5). Thus, aire does not dictate differentiation of MECs, nor their ability to present antigens.

To obtain a broad perspective on the transcriptional profile of MECs, we prepared RNA from cells sorted from aire-deficient and control littermates (sorting pooled thymocytes from five individuals as described in Fig. 2A) and used labeled complementary RNA to probe 12,000 mouse genes represented on Affymetrix U74Av2 chips (*31*). Three independent experiments were performed to permit statistical treatment of the data, and three different statistical assays were applied (*31*). The absence of aire resulted in loss of or reduction in expression of a substantial number of genes (Fig. 5A), clearly more than in the randomly permuted data set. When using a factor of 5 change as an arbitrary cutoff value, decreases in transcripts of 104 genes were called significant by the Significance Analysis of Microarrays (SAM) program and 42 by the Quadrants program; decreases ranged from a factor of 5 to a factor of 70, frequently with complete extinction in mutant mice. In contrast, the number of genes induced in mutant mice did not depart from that of the random data set (14 significant by SAM, 6 by Quadrants). Thus, aire seems to function predominantly as a transcriptional activator. Because of statistical uncertainties linked to multiple sampling, the exact number of genes represented on the U74Av2 chip and down-regulated in the absence of aire cannot be ascertained, but an estimate of 100 to 300 is a reasonable extrapolation, on the basis of the data in Figs. 5A and 6A and the statistical analysis. Similarly, it is not exactly certain what fraction of all murine transcripts are represented on the chip we used (likely ½ to ¼). Therefore, the number of genes whose expression is activated by aire probably ranges between 200 and 1200.

If aire controls autoimmunity by promoting ectopic expression of peripheral antigens, one would expect a strong reduction in those genes ectopically expressed in MECs of aire-deficient mice. This prediction was tested (Fig. 5B) using a previously reported list of ectopic transcripts (*4*). Most of these genes were silenced or at least significantly repressed in MECs derived from mutant animals. This observation was solidified by closer examination of the 30 genes most strongly down-regulated in mutant MECs (Fig. 6A). Strikingly, the vast majority were ectopically expressed transcripts of peripheral tissue–restricted antigens, normally associated with functions related to the terminal differen-

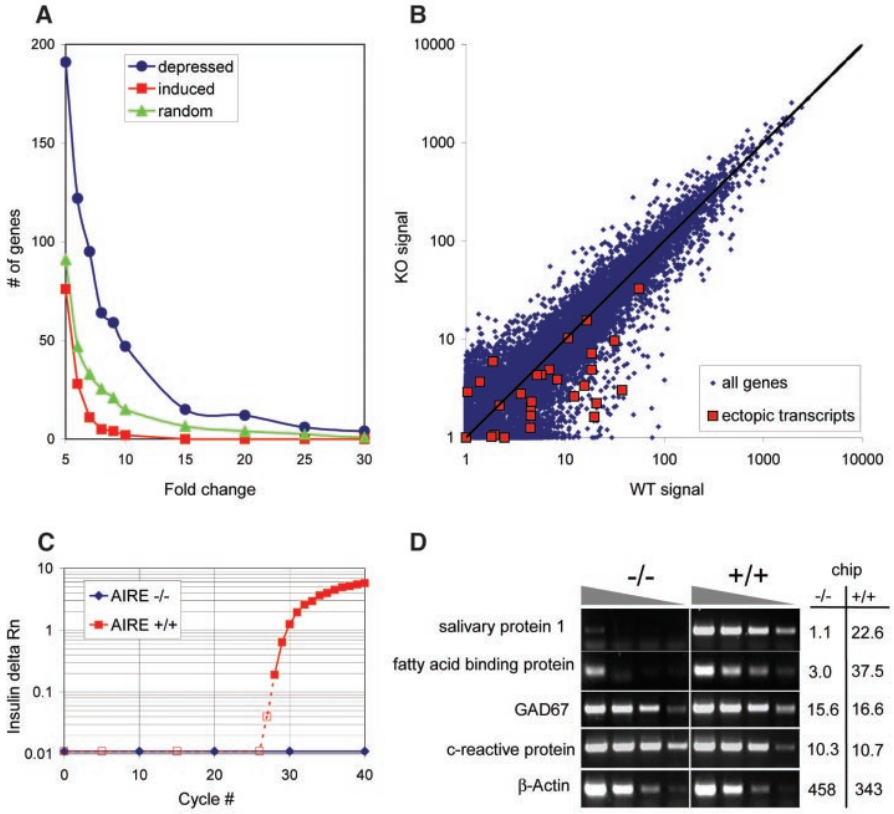

Fig. 5. Variation in gene expression in aire-deficient MECs. Affymetrix murine U74Av2 gene chips were hybridized with twice-amplified RNA (*31*) from knockout or wild-type thymic MECs. (**A**) The curves represent the cumulative number of genes showing the indicated relative changes in expression (fold change), either depressed (circles) or induced (squares). They are compared to a control curve derived from a randomly permuted data set (triangles). (**B**) Two-dimensional scatterplot of gene expression values for all genes on the chips (available at www.ncbi.nlm.nih.gov/geo, GEO series accession number GSE85), representing expression in MECs from wild-type (WT) or knockout (KO) mice. Red squares represent ectopic transcripts [described by (*4*)]. (**C** and **D**) Confirmation of chip expression data by real-time PCR of insulin expression (C) (normalized to cyclophilin), or by semiquantitative RT-PCR (D) (fourfold serial dilutions) of several tissue-specific genes compared with the relative expression values from the gene chips (right).

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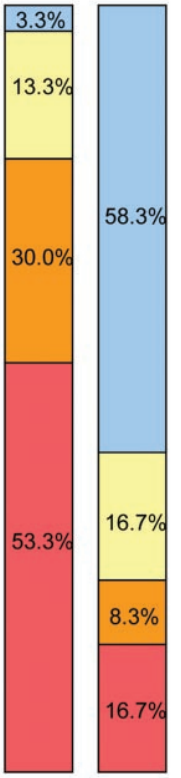
Fig. 6. AIRE primarily promotes transcription of tissue-specific genes. (**A**) The 30 genes most depressed in the aire knockout, ordered according to their expression change and color-coded according to their organismal expression patterns. These were determined with a combination of searches in the literature and of UniGene listings of expressed sequence tag distribution (NCBI). For each gene, the relative expression signal for wild-type and knockout is given, as well as the relative change in expression (KO/WT). Measures of significance included the *P* value, determined using Welch's approximate *t* test, and the false positive rate (FPR), the proportion of false positives expected at a particular level of difference between knockout and wild-type, determined using both the Quadrants (Quad) and Significance Analysis of Microarrays (SAM) programs. (**B**) The percentage of genes from (A) in each expression category compared with the percentage of genes in a random list in each category. The colors in (B) correspond to those in (A).

A

Probe name	Gene name	Tissue(s)	WT signal	KO signal	KO/WT	t-test p-val	FPR Quad	FPR SAM
96030_at	casein alpha	mammary	75.62	1	0.013	0.0417	0.043	0.014
97180_f_at	hemoglobin y, beta-like embryonic chain	fetal erythrocytes	87.36	1.29	0.015	0.0803	0.279	0.014
100106_at	intestinal trefoil factor	intestinal goblet cells	74.59	1.99	0.027	0.0504	< 0.02	0.014
101820_at	neurotoxin homologue	granulocytes, monocytes	29.50	1	0.034	0.1105	< 0.02	0.014
94738_s_at	cryptdin,related sequence 2	Paneth cells	100.47	3.85	0.038	0.2561	0.164	-
101682_f_at	major urinary protein IV	lachrymal gland, parotid gland	26.49	1.2	0.045	0.0392	0.063	0.014
94153_g_at	salivary protein 1	salivary gland	22.62	1.06	0.047	0.0712	0.131	0.014
102998_at	cytochrome P450 1a2	liver, lung, duodenum	20.99	1.01	0.048	0.0868	0.043	0.014
101115_at	lactotransferrin	mammary gland, uterus	19.78	1	0.051	0.0320	0.043	0.014
92353_at	serine protease (BSSP)	hair follicles, brain	18.77	1	0.053	0.1070	1	-
100463_at	gamma-casein precursor	mammary gland	21.93	1.17	0.053	0.0596	0.071	0.014
92546_r_at	prostaglandin D	brain, epididymis	22.82	1.26	0.055	0.0001	0.063	0.014
96153_at	neutrophilic granule	granulocytes	28.46	1.72	0.060	0.0246	0.063	0.014
160899_at	Purkinje cell protein 4	brain, eye (lens)	32.67	2.09	0.064	0.0327	< 0.02	0.014
161815_f_at	major urinary protein I	liver	31.23	2.04	0.065	0.0704	0.043	0.014
98858_at	glucose dependent insulinotropic polypeptide	K cells of small intestine	27.16	1.78	0.066	0.0517	< 0.02	0.014
101910_f_at	major urinary protein 3	liver	21.02	1.47	0.070	0.0328	0.164	0.014
94775_at	oxytocin	brain	26.59	1.92	0.072	0.0334	1	-
101636_at	salivary protein 2	salivary gland	16.80	1.23	0.073	0.0382	1	-
98623_g_at	insulin-like growth factor II	embryo, choroid plexus and leptomeninges in adult	94.85	6.96	0.073	0.1179	< 0.02	0.014
99958_at	mast cell protease-2	mast cells	13.70	1.01	0.074	0.0248	0.043	0.014
94707_s_at	amelogenin	ameloblast cells	34.69	2.57	0.074	0.0328	< 0.02	0.014
103235_at	preproneuropeptide y	brain	19.54	1.47	0.075	0.0143	0.043	0.014
103887_at	S100 calcium binding protein A9	immature BM myeloid cells, monocytes, neutrophils	68.93	5.26	0.076	0.1529	0.279	0.014
162341_r_at	aldose reductase	many	19.37	1.48	0.076	0.0121	0.279	-
97889_at	fatty acid binding protein	intestine	37.46	3.04	0.081	0.0254	0.043	0.014
94045_at	α-1-microglobulin/bikunin precursor	liver	12.74	1.04	0.082	0.0781	0.279	0.014
100150_f_at	preproinsulin II	pancreatic islet beta cells	19.70	1.62	0.082	0.1692	< 0.02	0.014
100002_at	inter-alpha-inhibitor H3 chain	liver, brain	12.07	1	0.083	0.0266	0.043	0.014
98830_at	spermine binding protein	prostate	13.56	1.13	0.083	0.1047	0.131	-

B



one specific tissue
several specific tissues
hematopoietic cells
housekeeping

tiation state of cells in particular organs. Only one transcript derived from a ubiquitously expressed gene (Fig. 6A). By comparison, a random sampling of genes from the array showed that 25% fell into the category of expression in rare tissues ("one specific/several specific tissues") and 58.3% into the ubiquitously expressed ("housekeeping") category (Fig. 6B). Note that the genes whose expression in MECs depended on aire included a significant number expressed in tissues targeted by the autoimmune attack (e.g., salivary proteins 1 and 2, zona pellucida glycoprotein 3). In addition, we found two examples of proteins (cytochrome P450 1A2 and preproinsulin) whose expression was abolished in MECs of mutant mice and that are recognized by autoantibodies from multiple APECED patients. This observation forges a strong link between aire control of MEC transcripts and of peripheral autoimmune attack (*23, 37*).

To verify the relative measurements of gene expression derived from the microarray analysis, we performed quantitative real-time and semiquantitative reverse-transcription PCR (RT-PCR) analyses on cDNA from sorted MECs from aire-deficient and control littermates (Fig. 5, C and D). The results were confirmatory for the genes examined, and the findings also serve to substantiate the observation that not all ectopic transcripts are dependent on aire expression. Although insulin, fatty acid–binding protein, and salivary protein–1 transcripts were absent or strongly reduced in MECs from mutant mice, genes encoding other proteins examined, such as C-reactive protein or glutamatic acid decarboxylase, were still expressed.

Discussion. These results raise several issues. First, aire seems to broadly control central tolerance induction, yet the spectrum of autoimmunity resulting from the loss of this transcription factor is quite defined. Not all peripheral organs are attacked, and within a particular organ only certain substructures are targeted. Interestingly, the defined autoimmune pattern shows many similarities to that observed in mice subjected to neonatal thymectomy (*38*). This pattern probably reflects a number of elements, including MHC restriction of antigens, antigen-presenting cell distributions within different organs, and blood-organ barriers. Also, ectopic transcription of different genes in thymic MECs is controlled by aire to variable degrees—some (e.g., casein-α, hemoglobin-γ) very strongly, others (trypsin 2) relatively weakly, and still others (C-reactive protein) not detectably. One or more other transcription factor(s) might have an activity similar to aire in regulating ectopic expression of peripheral antigens in MECs, or aire may be involved in a multimeric complex that displays partial activity in its absence.

Second, the molecular mechanism by which aire controls ectopic expression of a defined battery of genes specifically in MECs is intriguing from several perspectives. It seems rather unlikely that each of these several hundred genes has an aire-binding site such as the one described in (*25*). More likely, aire complexes with and influences the activity of multiple other transcription factors; indeed, it is already known to interact with the common coactivator CREB-binding protein (CBP) (*26*). Other possibilities involve broad regulatory processes at the gene or chromatin level, such as DNA methylation, histone acetylation, nucleosome assembly, or chromatin remodeling. Any proposed mechanism will also need to account for the observation that the ectopic expression of particular peripheral genes was restricted to a small subset of MECs (*5*). This latter point may underlie our observation that the specific substructures targeted for a particular organ can differ in different individuals—for the eye, autoantibodies directed at the rod and cone layer of the retina in

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one mouse (Fig. 1D), to other retinal layers in another mouse (*32*).

Finally, it is worth considering aire-deficient mice as a model of APECED. Like human patients, the murine model shows multiorgan attack by lymphocytic infiltrates and recognition by serum autoantibodies. In both cases, there is disease heterogeneity between individuals and exacerbation with age. The differences between humans and mice in the spectrum of organs targeted could well be due to the influence of genetic modifiers, as recently described for the HLA locus in humans (*39*). Aire-deficient mice should prove invaluable for dissecting the relative importance of genetic, environmental, and stochastic processes in determining the target organ specificity of autoimmune destruction.

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Figs. S1 to S8
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REPORTS

Broadband Modulation of Light by Using an Electro-Optic Polymer

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A major challenge to increasing bandwidth in optical telecommunications is to encode electronic signals onto a lightwave carrier by modulating the light up to very fast rates. Polymer electro-optic materials have the necessary properties to function in photonic devices beyond the 40-GHz bandwidth currently available. An appropriate choice of polymers is shown to effectively eliminate the factors contributing to an optical modulator's decay in the high-frequency response. The resulting device modulates light with a bandwidth of 150 to 200 GHz and produces detectable modulation signal at 1.6 THz. These rates are faster than anticipated bandwidth requirements for the foreseeable future.

Transmitting signals by using infrared light through optical fiber is the most effective way to move large amounts of data rapidly over long distances. Consequently, optical communications form the backbone of the Internet and telephone networks, and they are envisioned to carry real-time multimedia content in the future.

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Approaches to increasing optical bandwidth are being pursued to accommodate anticipated growth in data traffic. At present, high-speed optical networks use bandwidths of 10 GHz (~10 billion bits per second) per channel, and 40-GHz products are being introduced. Basic research efforts are aiming to push bandwidths to 80, 100, and even 160 GHz.

Expanding bandwidth beyond 100 GHz involves many scientific and engineering challenges, among which is the encoding of electronic data signals onto a lightwave carrier by modulating the light in phase or amplitude. This is usually done with an electro-optic (E-O) modulator (*1*), where a signal voltage changes the refractive index of an E-O dielectric optical waveguide, modulating the phase of a guided lightwave. Amplitude modulation is obtained by generating a phase difference $\Delta\Phi$ between light in two coherent waveguides and then combining to produce constructive or destructive interference. In present technology, lithium niobate is the E-O material most widely used in high-speed optical modulators.

An E-O device produces the strongest modulation when $\Delta\Phi = \pi$ radians. For an input voltage V_{in}, $\Delta\Phi \propto \pi(V_{in}/V_\pi)$, where V_π, the "half-wave" voltage that makes $\Delta\Phi = \pi$ near zero frequency [direct current (dc)], is:

$$V_\pi = \frac{\lambda_0 d}{2n_{opt}^3 r \Gamma L} \quad (1)$$

where λ_0 is the carrier wavelength, d is the gap between voltage electrodes, n_{opt} is the optical refraction index, r is the E-O coefficient of the dielectric, L is the device length, and Γ is defined as the signal electric field in the optical waveguide normalized to the field that would be there if air were the dielectric. The central problem for high-speed operation is that, for a fixed V_{in}, $\Delta\Phi$ decreases as modulation frequency increases. This response deterioration has three physical origins. The first is dissipation of V_{in} due to resistance in the electrodes guid-

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