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SUPPLEMENTAL MATERIALS AND METHODS

Western blotting

Eye tissue was homogenized on ice in a buffer containing 50 mM Tris, 2% SDS, 100 mM dithiothreitol, and 10% glycerol. The homogenates were boiled for 5 min and centrifuged at 13,000 rpm for 5 min. The extracts were separated by 12% SDS-PAGE gel and electrophoretically transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membranes were blocked with TBS-T (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk overnight at 4°C. Membranes were assembled in a multiscreen apparatus (Mini-PROTEAN II; Bio-Rad Laboratories) and incubated for 1 h with a 1:600 dilution of sera. After washing five times with TBS-T, the bound antibodies were reacted with horseradish peroxidase–conjugated goat anti–mouse (1:15,000; Bio-Rad Laboratories) for 1 h. Membranes were washed another five times with TBS-T, and reactivity was visualized with an enhanced chemiluminescence reagent (Pierce Chemical Co.) and autoradiography.

Immunofluorescence

7-μm cryostat sections were prepared from the eye of an adult RAG-deficient mouse (The Jackson Laboratory) to avoid interference from endogenous immunoglobulins, stained with 1:200 dilutions of sera from aire-deficient mice or control littermates, and counterstained with FITC-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). A rabbit-raised anti-IRBP antibody was used as a positive control. Slides were mounted with VectaMount (Vector Laboratories) containing DAPI for identification of nuclei.

Immunostaining

7-μm cryostat sections were prepared from the eyes of aire-deficient mice or littermate controls. Sections were fixed in acetone at −20°C for 10 min and air dried. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 10 min. Sections were blocked in a buffer containing 0.1 M Tris, pH 8, 0.05 M NaPhos, 0.3% Tween 20, and 3% normal goat serum for 1 h at room temperature. Primary antibodies were diluted in blocking buffer at a dilution of 1:50 for CD4 and CD8 and 1:100 for IgD and incubated for 1 h at room temperature. Slides were washed in PBS with 0.01% Tween 20 and incubated in horseradish peroxidase–conjugated donkey anti–rat antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:200 in blocking buffer. Immunoreactivity was visualized using DAB chromogen (Vector Laboratories), and slides were counterstained with hematoxylin (Sigma-Aldrich).

Thymic stroma preparation

Thymi from 4-wk-old aire wild-type or aire-deficient mice were removed and trimmed of fat and connective tissue. Small cuts into the capsule were made, and the thymi were gently agitated in 50 ml RPMI 1640 for 30 min at 4°C. Thymic fragments were manually dispersed via pipetting, recovered by settling, and digested with 0.125% collagenase D (Roche) with 0.1% DNase (Promega) in RPMI 1640 at 37°C. This digestion was repeated for a total of two times, and supernatants were retained. Remaining fragments were fully digested in 0.125% collagenase D, 0.1% DNase, and dispase (Roche) in RPMI 1640 for 30 min at 37°C. All supernatant fractions were pooled, and cells were collected by centrifugation at 400 *g* for 10 min. $CD45^+$ cells were removed by negative selection using CD45 microbeads (Miltenyi Biotec) and an AutoMACS instrument (Miltenyi Biotec). RNA was prepared from the $CD45^-$ fraction.

Microarray analysis

Both microarray studies were processed and normalized using MAS5.0 (Affymetrix). Only probes that were called present in all wild-type mice were considered. Locus-link IDs for genes that were repressed upon loss of aire were overlapped from both datasets; thus, genes had to be repressed in both studies ($P < 0.05$ using a *t* test). Next, the corresponding human locus-link ID was found for each mouse locus-link ID and with the retinal-specific gene list. A total of nine genes were both aire-dependent in the mouse and specific to the retinal compartment of the human eye.

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