Supporting Information

Taniguchi et al. 10.1073/pnas.1120607109

SI Materials and Methods

Tetramer Analysis and Flow Cytometry. Tetramers were generated by J.J.M. as described previously (1). Tetramer staining was performed following a protocol of Moon et al. (2). In brief, cells from the cervical lymph nodes, submandibular lymph nodes, axillary lymph nodes, inguinal lymph nodes, mesenteric lymph nodes, and spleen were pooled and stained with tetramer for 1 h at room temperature. Magnetic bead separation (MACS column; Miltenyi Biotech) was performed to enrich for tetramer-positive cells. The positively selected cells were stained with anti-CD3 (145-2C11; BioLegend), anti-CD4 (GK1.5; BioLegend), anti-CD8 (53-6.7; Biolegend), anti-B220 (RA3-6B2; eBiosciences), NK1.1 (PK136; BioLegend), F4/80 (CI:A3-1; Biolegend), CD11b (M1/70; Biolegend), CD11c (N418; Biolegend), and CD44 (IM7; Biolegend). Tetramer-reactive cells were gated on $CD3^+CD4^+CD8^-CD11b^-CD11c^-F4/80^-NK1.1^-$ lymphocytes. Tetramer-positive cells were quantified by FACS analysis using counting beads (CountBright; Invitrogen).

Histology. Organs from mice were harvested and fixed overnight in Fisher 10% Formalin [4% formaldehyde (wt/vol), 2% sodium acetate (wt/vol), 1.5% methanol (wt/vol)] embedded in paraffin, sectioned, and stained with H&E. Uveitis was identified based on the presence or absence of histological infiltrates and tissue disruption/damage in the retina.

Immunizations. Mice were immunized with 100 μg of IRBP(271-290) or IRBP(771-790) peptide emulsified in 100 μL of CFA (incomplete Freund's adjuvant plus 200 μg of heat-killed *Mycobacterium tuberculosis* strain H37Ra; Difco). For immunization with whole IRBP, eyes were homogenized in PBS. The eye suspension was vortexed on high for 2 min, then filtered through a 70-μm filter. The filtered suspension was centrifuged for 20 min at 1,800 × *g*, and the supernatant was filtered again through a 0.22-μm sterile filter. The IRBP was concentrated using spin tubes for >100-kDa proteins (Amicon Ultra-15 centrifuge tubes; Millipore). Then 100 μL of concentrated eye protein from eight eyes plus 100 μL of CFA were emulsified and injected s.c. into each mouse.

Hybridoma Generation. Hybridomas were generated according to standard protocols (3). In brief, $Aire^{-/-}$ C57BL/6 mice were immunized with either 100 μg of IRBP(771-790) or 250 μg of whole bovine IRBP (provided by R. Caspi, National Institutes of Health, National Eye Institute, Bethesda, MD) emulsified in 100 μL of CFA. After 10 d, cervical lymph node and spleen cells were isolated and stimulated with 20 mg/mL of whole bovine IRBP. Three days later, the cells were purified by Ficoll centrifugation and fused to BW1100 fusion partners with PEG1500 (Sigma-Aldrich). Fused cells were plated with 10E6/mL of thymocytes in DMEM supplemented with 20% FCS (DMEM-20c). Cells were aliquoted into 96-well plates (100 μL/well). At 1 d postfusion, the cells were selected on hypoxanthine-aminopterin-thymidine (Sigma-Aldrich) supplemented media. Cells were incubated on this media for 10 d, and clones that grew out were changed to hypoxanthine-thymidine (Gibco) in DMEM-20c. After 7 d, cells were grown on DMEM-20c.

Eye Antigen Preparation Containing IRBP. Eyes from WT or $IRBP^{-/-}$ mice were homogenized in RPMI supplemented with Hepes, sodium pyruvate, nonessential amino acids, penicillin/streptomycin, β2me, and 10% FBS (complete RPMI). The eye suspension was vortexed on high for 2 min and then filtered through a 70-μm filter. The filtered suspension was centrifuged for 20 min at 1,800 × *g*, and the supernatant was filtered again through a 0.22-μm sterile filter. A suspension of one eye per 3 mL of RPMI was designated as 25 "arbitrary units of murine eye antigen" and used to stimulate IRBP-specific hybridomas.

Thymic Dendritic Cell Sorting. Thymic dendritic cells were prepared for sorting by mincing thymi with surgical razor blades. Minced thymi were placed in complete RPMI containing 0.125% Collagenase D (Roche), 10 ug/mL of Dispase I (Roche), and 20 ug/mL of DNase I (Roche) and rotated in 37 °C for 40 min. Digested cells were then resuspended in PBS with 0.5% BSA and 2 mM EDTA, and passed through a 70-μm cell strainer. Density-gradient centrifugation using a three-layer Percoll gradient (GE Healthcare) with specific gravities of 1.115, 1.065, and 1.0 was used to enrich for the light fraction between the 1.065 and 1.0 layers. The light fraction was sorted on a FACSAria (BD Biosciences) for the $CD45^+CD11c^+$ antigen-presenting cells.

Hybridoma Stimulation Assay. Hybridomas were incubated with syngeneic antigen-presenting cells plus the indicated concentration of antigen. After 48 h or 15 h of stimulation, hybridomas were analyzed by flow cytometry for up-regulation of CD69 (clone H1.2F3; Biolegend).

1. Moon JJ, et al. (2007) Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27:203–213.
2. Moon JJ, et al. (2009) Tracking epitope-specific T cells. *Nat Protoc* 4:565–581.
3. Kruisbeek AM (2001) Production of mouse T cell hybridomas. *Curr Protoc Immunol* 24: 3.14.1–3.14.11.

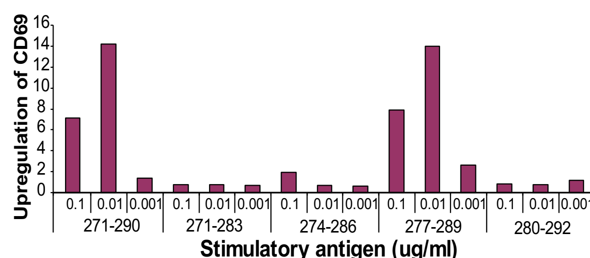

Fig. S1. LB4 is activated by P2. The LB4 hybridoma was stimulated with irradiated WT B6 splenocytes and the indicated IRBP-derived peptides. After 12 h, TCR stimulation was measured by CD69 up-regulation.

Peptide name	Amino acid position	Peptide design	Sequence	H-2 I-A^b binding (IC50 nM)
P2	IRBP: 271-290	T cell hybridoma stimulation	PLGGGGQTWE GSGVLPCVGT	294
P7	IRBP: 771-790	High affinity for I-A^b predicted by IEDB	SYSSAVPLLC SYFFEAEPRQ	51

Fig. S2. P2-IAb and P7-IAb tetramer reagents bind antigen-specific T cells. IRBP 277–290 (P2) and IRBP 771–790 (P7) peptides loaded onto I-Ab were used to produce MHC class II tetramer reagents. The affinity of P2 and P7 peptides for I-Ab was measured by determining the concentration of free P2 or P7 peptide needed to displace 50% of radiolabeled P2 or P7 peptide preloaded on IAb (IC_{50}).

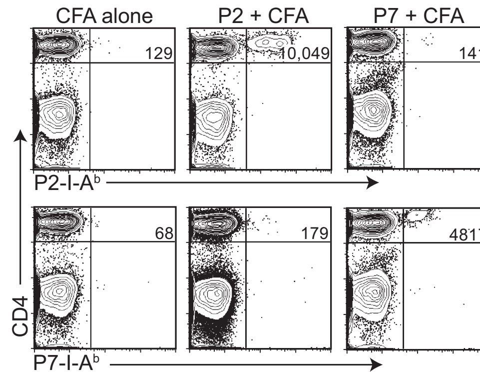

Fig. S3. P2-IAb and P7-IAb tetramer reagents bind antigen-specific T cells. IRBP$^{-/-}$ mice were immunized with CFA alone, with P2 peptide in CFA, or with P7 peptide in CFA. At 8 d after immunization, spleen and peripheral lymph nodes were pooled from individual mice and stained with the P2-I-Ab and P7-I-Ab tetramers. The tetramer-binding cells were enriched via magnetic bead separation and analyzed by flow cytometry. Representative FACS plots of tetramer-enriched peripheral CD3$^+$ T cells are shown. The absolute number of tetramer-positive cells from one mouse is noted in the FACS plots. Data are representative of at least three sets of experiments.

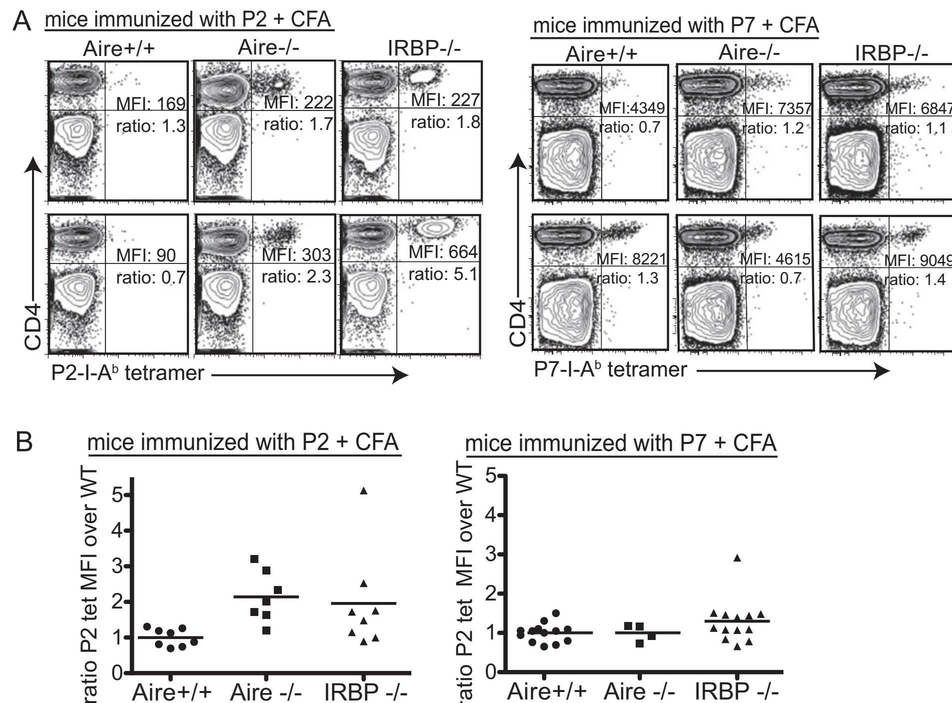

Fig. S4. T cells expanded in Aire$^{-/-}$ mice have greater avidity for P2 than those expanded in Aire$^{+/+}$ mice, whereas P7-specific cells in Aire$^{-/-}$ and Aire$^{+/+}$ mice have similar avidity for P7. Four- to 7-wk-old Aire$^{+/+}$, Aire$^{-/-}$, and IRBP$^{-/-}$ mice were immunized with either P2 peptide or P7 peptide in CFA. At 8 d after immunization, the tetramer mean fluorescence intensity (MFI) of tetramer-positive T cells in the secondary lymphoid organs was analyzed (same mice as shown in Fig. 4). (*A*) Representative data from one experiment: FACS plots of 12 mice analyzed on the same day, with tetramer MFI values in the upper right quadrant. (*B*) Relative MFIs from several experiments run on different days. To normalize the tetramer MFI for experiment-to-experiment variations inherent in flow cytometry analysis, the MFIs of tetramer-positive cells of individual samples were divided by the mean tetramer MFI from Aire$^{+/+}$ mice run on the same day. This ratio is given as "tet MFI over WT." Thus, a ratio of 1 means that cells from that sample have the same MFI as the average MFI of Aire$^{+/+}$ T cells run on the same day, whereas a ratio >1 means that cells from that sample have a higher tetramer MFI than the average MFI of Aire$^{+/+}$ T cells.

Frequency of uveitis after immunization

Immunization	Aire +/+	Aire -/-
P2 + CFA	0/5	5/6
P7 + CFA	0/5	2/6
none	ND	0/4

Fig. S5. Uveitis can be induced by immunizing young Aire$^{-/-}$ mice with P2 or P7 peptide in CFA. Four- to 5-wk-old Aire$^{+/+}$ and Aire$^{-/-}$ mice were immunized with either P2 peptide or P7 peptide in CFA. At 3 wk after immunization, the mice were killed, and the onset of uveitis was identified by histology. The data indicate the number of mice with uveitis over the number of mice analyzed. All mice shown were age 7–8 wk at the time of analysis, an age at which no spontaneous uveitis can be observed in Aire$^{-/-}$ mice (1). ND, experiment not done.

PNAS PNAS PNAS PNAS