A Specific Anti-Aire Antibody Reveals Aire Expression Is Restricted to Medullary Thymic Epithelial Cells and Not Expressed in Periphery[1]

François-Xavier Hubert,[2]*† Sarah A. Kinkel,*†‡ Kylie E. Webster,[3]* Ping Cannon,* Pauline E. Crewther,* Anna I. Proeitto,† Li Wu,† William R. Heath,† and Hamish S. Scott[2,4]*‡

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy is an autoimmune disorder caused by mutations in the autoimmune regulator gene AIRE. We examined the expression of Aire in different organs (thymus, spleen, and lymph nodes) in C57BL/6 mice, using a novel rat mAb, specific for murine Aire. Using flow cytometry, directly fluorochrome-labeled mAb revealed Aire expression in a rare thymic cellular subset that was CD45$^-$, expressed low levels of Ly51, and was high for MHC-II and EpCam. This subset also expressed a specific pattern of costimulatory molecules, including CD40, CD80, and PD-L1. Immunohistochemical analysis revealed that Aire$^+$ cells were specifically localized to the thymus or, more precisely, to the cortico-medulla junction and medulla, correlating with the site of negative selection. Although in agreement with previous studies, low levels of Aire mRNA was detected in all dendritic cell subtypes however lacZ staining, immunohistochemistry and flow cytometry failed to detect Aire protein. At a cellular level, Aire was expressed in perinuclear speckles within the nucleus. This report provides the first detailed analysis of Aire protein expression, highlighting the precise location at both the tissue and cellular level. ***The Journal of Immunology,*** **2008, 180: 3824–3832.**

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED),[5] also known as autoimmune polyglandular syndrome type I (APS I), is an autosomal recessive disorder. It is the only organ-specific human autoimmune disease described that affects multiple organs. APECED is characterized by various circulating tissue-specific autoantibodies that cause the destruction of the target organs, mainly the various endocrine glands. Patients also manifest chronic mucocutaneous candidiasis and a wide spectrum of ectodermal dystrophies. The cardinal symptoms in APECED are hypoparathyroidism, primary adrenocortical failure and chronic mucocutaneous candidiasis (1). This disease is caused by mutations in the autoimmune regulator (AIRE) gene encoded on chromosome 21q22.3 (2). AIRE is an important mediator of central tolerance that promotes the "promiscuous" expression of organ-specific Ags in the thymus, which is associated with the negative selection of autoreactive T cells (3–6).

Although the literature concurs that AIRE/Aire is expressed in thymic epithelial cells (TECs), it has also been reported that AIRE/Aire is expressed in lymph nodes, in the peripheral monocyte/dendritic cell lineage (7–10) and recently, in a subset lymph node stromal cells (11). However, AIRE/Aire expression in the periphery remains controversial and moreover, the significance of AIRE/Aire in the peripheral immune responses is as yet poorly understood.

The present study describes the generation of a mouse Aire-specific rat mAb named 5H12. This mAb, directly conjugated, can distinguish Aire$^+$ cells from total cells ex vivo by flow cytometry and reveals that Aire expression is restricted to the thymic medulla compartment and specifically to mature mTECs: CD45$^-$ MHC-II^{high} Ly51^{low}. This Ab also allows us to estimate the proportion of Aire-expressing mTECs in the thymus and define a more precise mTEC phenotype. In vivo, at a nuclear level, Aire expression is restricted in the perinuclear region, as nuclear dots.

Materials and Methods

Mice

All mice C57BL/6 (B6), OT-I, and RIP-OVA^{high} were bred and maintained at the Walter and Eliza Hall Institute for Medical Research. C57BL/6 mice deficient for Aire gene were generated via homologous recombination of targeting vectors in mouse C57BL/6 embryonic stem (ES) cells. Insertion of the Aire targeting vector disrupted exon 8 and brought the LacZ reporter gene under the control of the endogenous Aire promoter, creating an Aire-LacZ fusion. A phosphoglycerate kinase neomycin (PGK-Neo) cassette was used to select positive recombination events and was later removed using the flanking LoxP sites and Cre recombinase. All C57BL/6 and Aire-deficient mice were used at 6- to 10-wk-old. All studies were performed according to protocols approved by the Melbourne Health Animal Ethics Committee.

*Division of Molecular and Medicine and †Division of Immunology, Walter and Eliza Hall Institute of Medical Research, and ‡Department of Medical Biology, University of Melbourne, Parkville, Victoria, Australia

Received for publication July 13, 2007. Accepted for publication January 4, 2008.

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[1] This work was supported by fellowships from la Fondation pour la Recherche Medicale (to F.-X.H.), Australian Postgraduate Awards (to K.E.W. and S.A.K.), Howard Hughes Medical Institute international scholar (to W.R.H.), National Health and Medical Research Council fellowships (171601 and 461204), National Health and Medical Research Council program grants (257501 and 264573), Eurothymaide, 6th Framework Programme of the European Union, and the Nossal Leadership Award from the Walter and Eliza Hall Institute of Medical Research (to H.S.S.).

[2] Address correspondence and reprint requests to Dr. François-Xavier Hubert and Dr. Hamish Scott, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia. E-mail addresses: hubert@wehi.edu.au and hamish.scott@imvs.sa.gov.au

[3] Current address: Immunology and Inflammation, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia.

[4] Current address: Division of Molecular Pathology, Institute of Medical and Veterinary Science and The Hanson Institute, Box 14 Rundle Mall Post Office, Adelaide, South Australia 5000 and School of Medicine, University of Adelaide, South Australia 5005, Australia.

[5] Abbreviations used in this paper: APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; APS I, autoimmune polyglandular syndrome type I; AIRE, autoimmune regulator; TEC, thymic epithelial cell; ES, embryonic stem; PGK-Neo, phosphoglycerate kinase neomycin; SIRP, signal regulatory protein; cTEC, cortical thymic epithelium; mTEC, medullar thymic epithelial cell; DC, dendritic cell.

Reagents

Abs purchased from BD Pharmingen include: FITC-conjugated anti-Ly51 (clone 6C3), PE-conjugated anti-MHC-II I-$A^{b,d,q}$ I-$E^{d,k}$ (clone M5/114.15.2), biotin-conjugated anti-Ly51 (clone 6C3), allophycocyanin-conjugated anti-CD45 (clone 30F11), and streptavidin-PerCp5.5 used to reveal biotin staining. Biotin-conjugated mAb include: anti-CD40 (clone FGK 45.5), anti-CD80 (clone 16–10A1), anti-CD86 (clone GL1), anti-PD-L1 (clone M1H5), anti-B7 H2 (clone HK5.3), and anti-PD L2 (clone Y25) (eBioscience) were provided by Ken Shortman (The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia). The following biotin-conjugated mAb anti Vcam-1 (clone MVCAM.A) was purchased from eBiosciences and anti-Epcam (clone G8.8) was provided by R. Boyd (Monash Immunology and Stem Cell Laboratories, Clayton, Victoria, Australia). P. Peterson (Molecular Pathology, Biomedicum, Tartu University, Estonia) supplied the rabbit polyclonal anti-Aire Ab.

Aire Ab

Rat anti-Aire mAb was produced in the mAb Facility at the Walter and Eliza Hall Institute by standard polyethylene glycol fusion. The selected hybridoma 5H12 was generated to a 21-amino acid peptide corresponding to the 20 COOH-terminal amino acids of Aire with a C residue at the NH2 terminus for KLH conjugation (CILQWAIQSMSRPLAETPPFSS).

Cell preparations

DCs were isolated essentially as previously described (12). In brief, spleen, thymus or lymph node fragments were digested for 25 min at room temperature with collagenase/DNase and then treated for 5 min with EDTA to disrupt T cell-DC complexes. Cells were recovered from the digest by centrifugation, the pellet was resuspended in a 1.077g/cm3 isoosmotic Nycodenz medium, centrifuged at 1700 rpm for 10 min and then the low-density fraction was collected. For cell sorting, cells were incubated with rat mAbs against mouse CD19 (ID3), CD3 (KT31.1), Thy1.1 (T24/31.7), TER-119, and Ly6G (1A8), and non-DCs depleted using anti-rat Ig magnetic beads (Qiagen). DCs were stained with varying combinations of mAbs to CD11c (N418-A594 or allophycocyanin), CD45RA (14.8-PE), CD11b (M1/70-Cy5), CD24 (M1/69-FITC or biotin), CD4 (GK1.5-allophycocyanin), CD8 (YTS169.4-FITC, PE, or allophycocyanin), signal regulatory protein (SIRP)-α (p84-biotin) and Ly5.2 (S450–15.2-FITC), with second-stage staining with streptavidin-PerCP-Cy5.5 or streptavidin-PE. Cell sorting and analysis was performed on a FACSVantageSE DiVa, FACStarPlus, LSR (BD Biosciences), or MoFlo (DakoCytomation) instruments.

mTECs were enriched from the thymus as previously described (13). In brief, thymi were collected into MT-RPMI, following a brief agitation using a wide bore glass pipette, the sample was then subjected to enzymatic digestion. Thymic fragments were incubated in 5 ml of 0.125% (w/v) collagenase D with 0.1% (w/v) DNase I (Boehringer Mannheim) in MT-RPMI at 37°C for 15 min. Cells released into suspension were removed after larger thymic fragments had settled. This was repeated three to four times with fresh media. In the final digest colllagenase D was replaced with trypsin. Each cell isolation was counted and the final two or three enrichments pooled. A negative depletion was performed to enrich for $CD45^-$ cells using CD45 microbeads (Miltenyi Biotec) and the AutoMACS system (Miltenyi Biotec), as per the manufacturer's instructions.

Flow cytometric analysis

Fluorescent-labeled preparations were analyzed on a FACScalibur instrument (BD Biosciences). Color compensation was checked using appropriate stained cell controls. Four fluorescent channels were used for the immunofluorescence staining (FL1 for FITC, FL2 for PE, FL3 for PerCP5.5, and FL4 for allophycocyanin). mTEC preparations were labeled with an anti-CD45, anti-MHC-II and an anti-Ly51. Then DC preparations were labeled with anti-MHC-II and anti-CD11c before fixation and permeabilization. The Aire intracellular staining was performed according to manufacturer's instructions in the BD Cytofix/Cytoperm kit.

Immunofluorescence

Thymi frozen in OCT compound (Tissue Tek) were freshly sectioned at 5 μm and stained with primary Abs: polyclonal rabbit anti-bovine cytokeratin (DakoCytomation) or UEA biotin (Vector Laboratories), Alexa 488 conjugated anti-Aire or purified anti-Aire diluted in MT-PBS/2% FCS in humid chamber for 30 min at room temperature. The sections were then washed twice in MT-PBS for 5 min, followed by 20 min incubation with the secondary Ab, a rhodamine goat anti-rabbit IgG (Santa Cruz Biotechnology) or a streptavidin FITC (BD Pharmingen) or a Alexa 546 goat anti-rat Ig (Molecular Probes). After washing, sections were stained with Hoechst 33342 (Sigma-Aldrich) for 5 min and washed a further three times. Sections were mounted and cover-slipped using DakoCytomation Fluorescent Mounting Medium (DakoCytomation) before viewing with confocal or fluorescent microscopy.

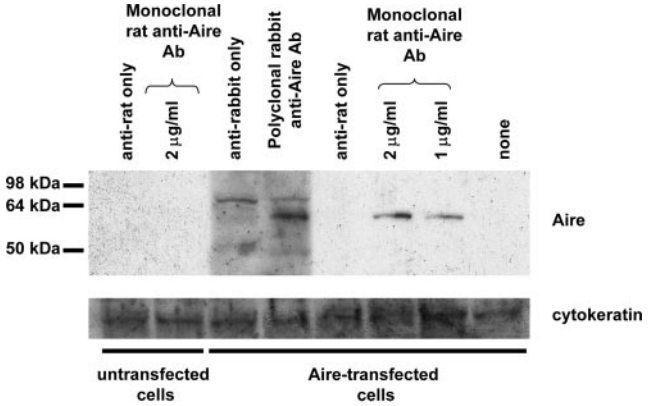

FIGURE 1. The specificity of the retained clone (here clone 12 named 5H12) was checked by Western blot on Aire transfected cells and compared with an anti-Aire polyclonal Ab. COS cells were electroporated with full-length mouse Aire in expression vector pcDNA3-myc-His (Invitrogen Life Technologies) or an empty vector for the negative control, referred to as untransfected cells. Secondary Abs, HRP-conjugated donkey anti-rabbit IgG or HRP-conjugated goat anti-rat IgG, were used to reveal specifically rat anti-Aire and rabbit anti-Aire, respectively.

X-gal staining

Tissues were taken from mice, immediately embedded in Tissue Tek-OCT compound (Sakura) and snap frozen in isopentane/liquid nitrogen. Cryostat sections at 7-μm thickness were thaw mounted onto polysine microscope slides and fixed in 4% paraformaldehyde in phosphate buffer (0.1 M; pH 7.6) at room temperature for 5 min. The slides were washed three times in wash buffer (phosphate buffer, 0.1 M (pH 7.6), containing 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, 2 mM $MgCl_2$) and stained in reaction buffer (wash buffer containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal) overnight at 37°C. The reaction was stopped by immersion in wash buffer followed by counterstaining with Nuclear Fast Red.

Real time PCR and quantitative real-time PCR

RNA was extracted from sorted mTECs and DCs using the RNeasy Micro Kit, including an on column DNase digest (Qiagen). Nested RT-PCR was performed using the following primers: Aire first round: For 5′-ggtccagcaccaagcgcgcgat-3′, Rev 5′-ccttgcatctctacaaagatcagg-3′; Aire second round: For 5′-tctgcagactcgactcccacgcca-3′, Rev 5′-tctggatggcc cactgcaggatgc-3′; Actb: For 5′-gacggccaggtcatcactattg-3′, Rev 5′-ctcat cgtactcctgcttgctg-3′. A 60-50°C touchdown PCR cycle was used. QRT-PCR was performed using Universal Probe Library chemistry (Roche) on the LC480 LightCycler (Roche) according to the manufacturer's protocol. Assays were designed to different regions of the Aire mRNA transcript (5′, middle, and 3′). The primers and probes used were as follows: Aire 5′: For 5′-gtccctgaggacaagttcca-3′, Rev 5′-gtcaggagcca ggacagc-3′, UPL probe 97; Aire middle: For 5′-ggttcctccccttccatc-3′, Rev 5′-ggcacactcatcctcgttct-3′, UPL probe #45; Aire 3′: For 5′-tgctagtcacgac cctgttct-3′, Rev 5′-ggatgccgtcaaatgagtg-3′, UPL probe 109.

Western blot analysis

The 5H12 mAb was tested at various concentrations by Western blot. COS cells were electroporated with full-length mouse Aire in expression vector pcDNA3-myc-His (Invitrogen Life Technologies) or an empty vector for the negative control. Cell lysates were made 16 h later using lysis buffer containing 1% SDS, 10% glycerol, 10% 2-ME, 40 mM Tris (pH 6.8), and 0.01% bromphenol blue. Equal amounts of cell lysate were run on a 12% Tris-glycine polyacrylamide gel (Invitrogen Life Technologies) and then transferred to a Hybond-ECL Western membrane (Amersham) at 50 V for 40 min. The membrane was blocked for 1 h in a 5% milk solution in PBS and was stained with primary Ab diluted in PBS/0.1% Tween20 and 5% milk for 2 h at 4°C. The membrane was washed with PBS and 0.1% Tween 20, incubated with secondary Ab, HRP-conjugated donkey anti-rabbit IgG

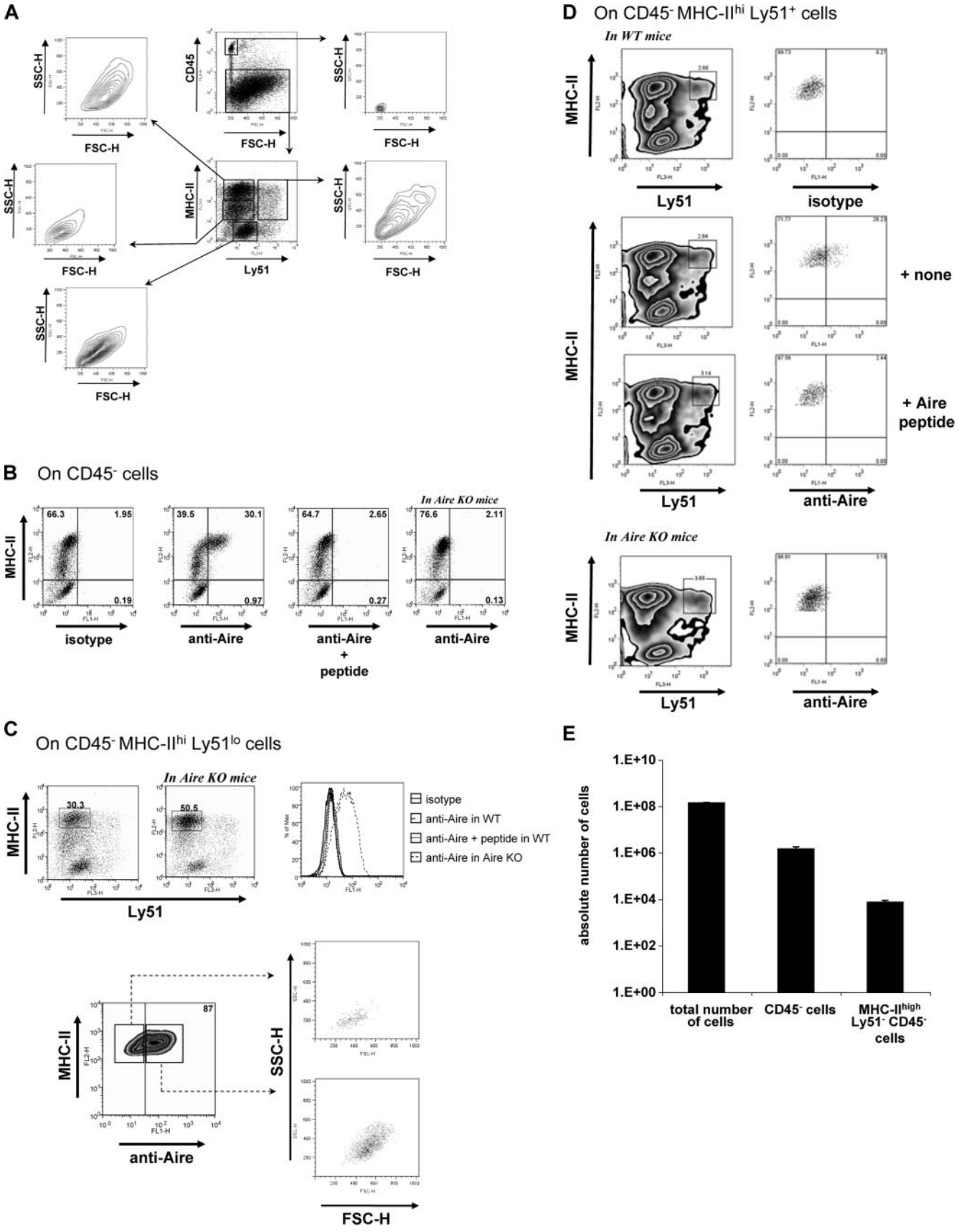

FIGURE 2. Aire is expressed by a group of nonhemopoietic thymic cells which are CD45$^-$ MHC-II^{high} Ly51^{low}. A negative depletion was performed to enrich for CD45$^-$ thymic cells from 6 to 10 wk old mice and the final two or three digested enrichments were pooled and analyzed for their expression of CD45 MHC-II, Ly51, and Aire. One representative experiment of four is shown. *A*, After gating on CD45$^-$ cells, mTECs (rectangle) are distinguished from others cell subsets according to their expression of MHC-II and Ly51. Different subsets were plotted according to their size (FSC) and granularity (SSC). *B*, Staining of CD45$^-$ cells for MHC-II and Aire revealed that only MHC-II^{high} cells expressed Aire. The specificity of the staining was tested after

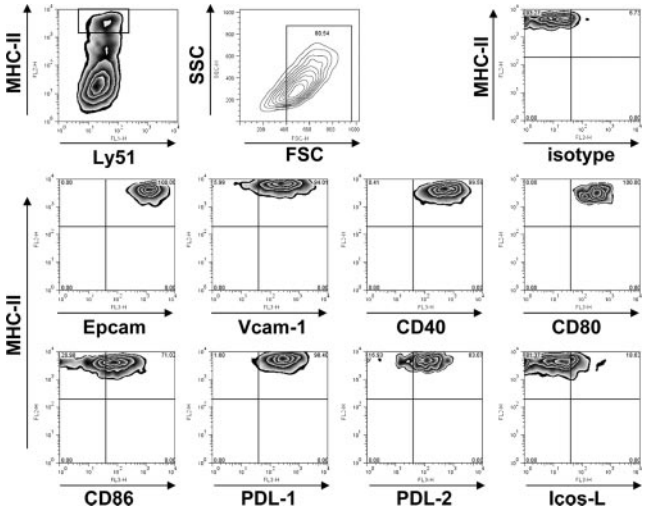

FIGURE 3. Costimulatory molecule expression on CD45$^-$ MHC-II^{high} Ly51^{low} cells. A negative depletion was performed from a pool of 6–8 mice aged 8–10 wk old to enrich for CD45$^-$ cells on the final 2 or 3 digested enrichments were pooled and analyzed for their expression of CD45, MHC-II, Ly51, and different costimulatory molecules after exclusion of smaller cells. Representative of two independent experiments.

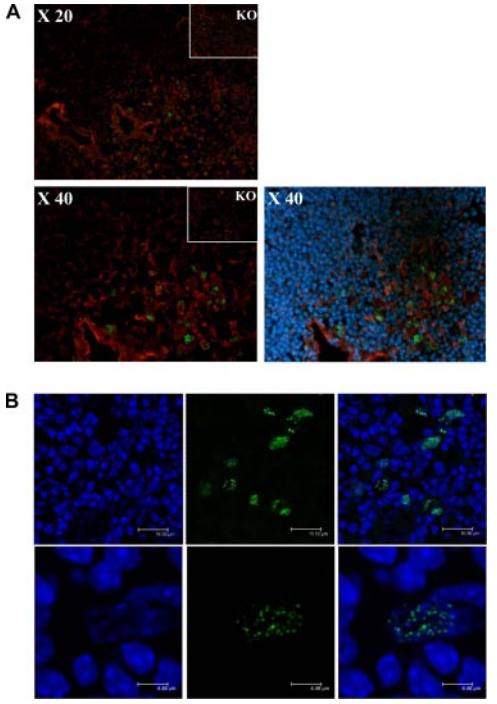

FIGURE 4. Aire immunofluorescence on thymic sections. *A*, Tissues from wild-type and Aire-deficient mice were stained with an anti-Aire Ab (green), an anti-keratin Ab (red) and Hoechst (blue). The immunofluorescence was visualized by fluorescent microscopy at magnification ×20 and ×40. *B*, Tissues from wild-type mice were stained with an anti-Aire Ab (green) and Hoechst (blue). The immunofluorescence was visualized by laser scanning confocal microscopy. First row is representative of Aire expression in medulla area, the second row is representative of Aire specific expression in one cell.

(Amersham Biosciences) or HRP-conjugated goat anti-rat IgG (Molecular Probes). Detection of Aire staining was performed using ECL Western blotting detection reagents (Amersham Biosciences) according to manufacturer's instructions.

CFSE-labeled T cell transfer

Lymph nodes (inguinal, axillary, cervical, sacral and mesenteric) were obtained from OT-I transgenic mice and CD8$^+$ T cells purified using a mixture of optimally tittered Abs to deplete cells expressing Mac-1 (M1/70), Mac-3 (F4/80), TER-119, GR-1 (RB6–8C5), MHC-II (M5/114), and CD4 (GK1.5), followed by sheep anti-rat Dynabeads (Dynal Biotech). Enriched cells contained 90–96% specific TCR transgenic CD8$^+$ T cells as determined by flow cytometry. These were labeled with CFSE (Molecular Probes); by incubating 10^7 purified cells/ml with 5 μM CFSE for 10 min at 37°C. Cells were then washed three times in HEPES modified Eagle's medium containing 2.5% FCS. CFSE-labeled T cells (10^6) were transferred to C57BL/6, RIP-OVA^{high} Aire$^{+/+}$ or RIP-OVA^{high} Aire$^{-/-}$ recipients i.v. 60 h posttransfer, lymphoid tissues were collected for analysis. Quantified T cell proliferation was assessed by CFSE dilution using flow cytometry.

Results

Selection of mAb 5H12 reactive against Aire$^+$ cells

To study Aire protein expression, we generated an Aire-specific Ab referred to as 5H12 (see *Materials and Methods*), which was of the IgG2c isotype (data not shown). To confirm its specificity, 5H12 was compared with a polyclonal rabbit anti-Aire Ab by Western blot on Aire-transfected cells. This Ab distinguish a single band of just under 64 KDa (Fig. 1), corresponding to the predicted m.w. of Aire (around 58 KDa), and of the same size a unique band detected by polyclonal anti-Aire rabbit serum.

mAbs 5H12 is highly reactive with Aire$^+$ cells

Thymic stromal cells, which include epithelium, endothelium, fibroblasts, and neuroendocrine cells, can be distinguished from cells of hemopoietic origin by their lack of expression of CD45 (Fig. 2*A*). Staining of CD45$^-$ cells for MHC-II and Aire (Fig. 2*B*), revealed that only MHC-II^{high} cells expressed Aire, with Aire-positive cells representing ~20% ± 15 of CD45$^-$ cells. To confirm the specificity of 5H12 mAb for Aire, staining was shown to be inhibited by the peptide used for rat immunization (Fig. 2*B*, third column) and was absent when the thymic stroma was derived from Aire$^{-/-}$ mice (last column in Fig. 2*B*). It has been established that cortical and medullary epithelial cells can be identified according to the expression of MHC-II (14) and Ly51 (13, 15), with both populations expressing high levels of MHC-II but only cortical thymic epithelium (cTEC) expressing Ly51 (Fig. 2*C*). After gating on CD45$^-$ MHC-II^{high} Ly51^{low} cells, which represent mature mTEC, virtually all cells (88% ± 4.8) were shown to express Aire (Fig. 2*C*).

To more precisely define the phenotype of Aire$^+$ cells, we examined their forward and side light scatter as a measure of relative cell size and granularity, respectively. Relative to CD45$^+$ cells, mature mTECs (MHC-II^{high} Ly51^{low}) and immature mTECs (MHC-II^{low} Ly51^{low}) were 2.5 times and 1.5 times larger, respectively (Fig. 2*A*), with mature mTECs having the highest granularity. In comparison, MHC-II$^-$ Ly51^{low} (fibroblast and endothelial

blocking with Aire peptide used for rat immunization as well as in Aire-deficient mice (*C*) Aire expression is analyzed on CD45$^-$ MHC-II^{high} Ly51^{low} cells and is represented on histogram and dot plot. The morphology of Aire positive or negative cells were analyzed for their size (FSC) and granularity (SSC). *D*, Aire expression on CD45$^-$ MHC-II^{high} Ly51^{high} cells corresponding to the cortical epithelial cells (cTECs). The specificity of the staining was tested after blocking with Aire peptide used for rat immunization as well as in Aire-deficient mice. *E*, Estimation of the number of CD45$^-$ cells and CD45$^-$ MHC-II^{high} Ly51^{low} cells in single 6-wk-old thymus. Each single thymus was digested and stained to analyze their expression of CD45, MHC-II, and Ly51. Sample data from 2×10^4 CD45$^-$ cells were acquired in duplicate on a FACSCalibur and results are expressed on a logarithmic *y* axis as mean ± SD of four mice.

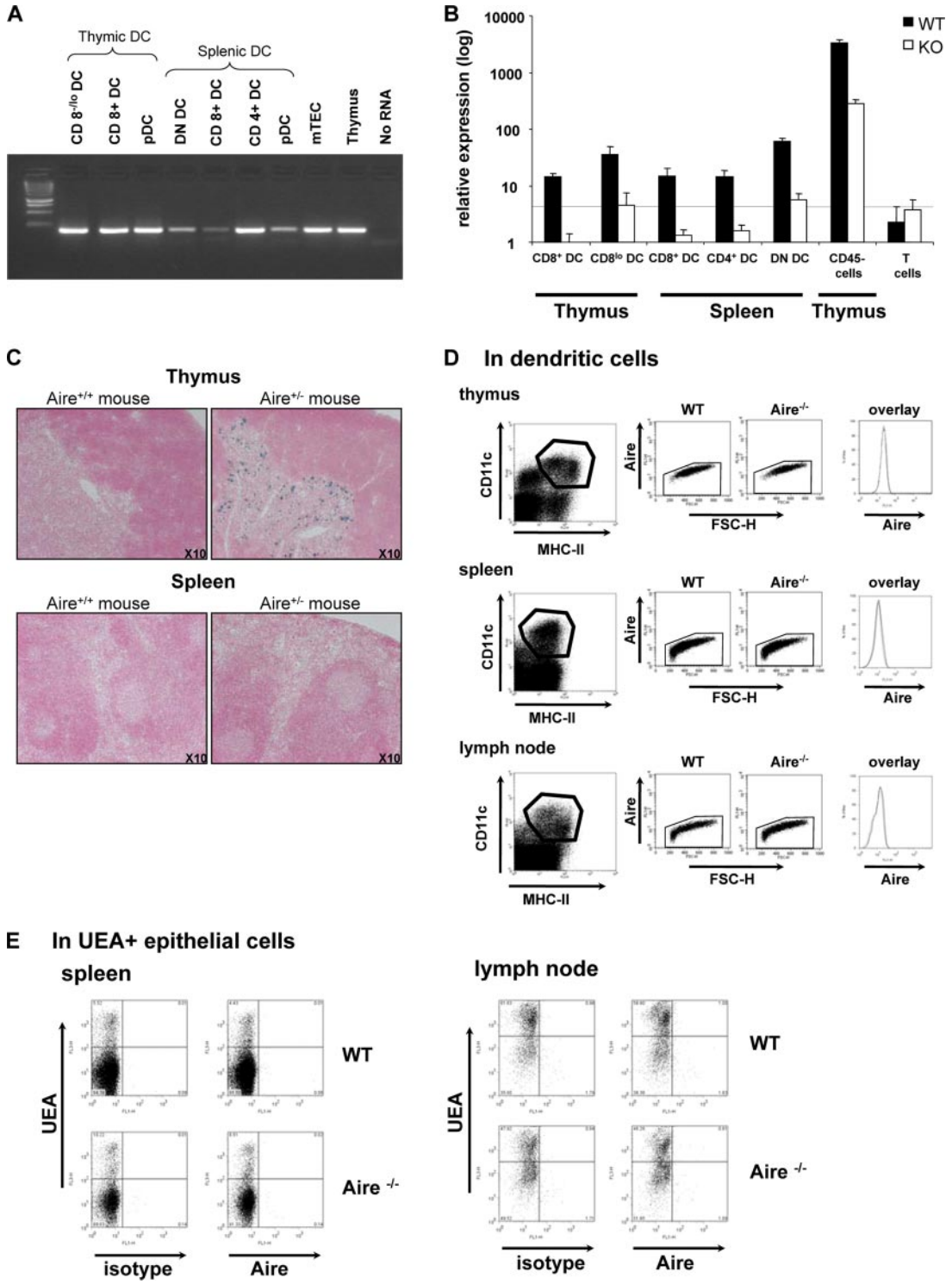

FIGURE 5. Presence of Aire mRNA expression in different thymic and splenic dendritic cell subsets but lack of Aire protein expression in spleen and lymph node. *A*, Spleen and thymus were digested, enriched by Nycodenz, depleted in non-DCs and DCs were sorted according to their expression of CD11c, CD45RA, CD11b, CD4, CD8, and SIRP-α. Aire mRNA expression was analyzed by nested PCR. *B*, Aire mRNA expression in different thymic and splenic dendritic cell subsets by real time quantitative PCR. Spleen and thymus were digested, enriched by Nycodenz, depleted in non-DCs, and DCs were sorted according to their expression of CD11c, CD45RA, CD11b, CD4, CD8, and SIRP-α. *C*, Aire is expressed in medullar thymic area but not in the spleen. X-gal staining detected expression of the lacZ reporter gene in nucleus of thymic Aire$^{+/-}$ mice but not in the spleen of Aire$^{+/+}$ and Aire$^{+/-}$ mice. Representative of two independent experiments with 2 sections/organs. *D*, Lack of Aire expression in dendritic cells from thymus, spleen and lymph node After digestion and enrichment of DC by Nycodenz, DCs were gated according to their expression of MHC-II and CD11c. Sample data from 5×10^4 MHC-II$^+$ CD11c$^+$ cells were acquired on a flow cytometer and were analyzed for Aire expression. Representative of five experiments. *E*, Lack of Aire expression in CD45$^-$ epithelial cells from spleen and lymph node. After digestion and depletion of hemopoietic cells, CD45$^-$ cells were stained according to their expression of Aire and UEA. Sample data from 4×10^4 CD45$^-$ cells were acquired on a flow cytometer. Representative of three experiments.

cells) and MHC-II$^+$ Ly51$^+$ (cTECs) showed a wide spread of size and granularity. When we analyzed the morphology of Aire$^+$ cell in the CD45$^-$ MHC-II^{high} Ly51^{low} mTEC population (Fig. 2*C*), this revealed that Aire$^-$ cells were smaller and less granular than Aire$^+$ cells.

Surprisingly, after gating on MHC-II$^+$ Ly51$^+$, normally corresponding to cTECs, between 10 to 20% were detected as Aire$^+$ cells (Fig. 2*D*). This represents only ~0.5% of total CD45$^-$ cells. These results suggest that either Aire is present in cTECs or that a small minority of mTEC express Ly51. The specificity of the staining was confirmed after blocking with Aire peptide (Fig. 2*D*), as well as in Aire-deficient mice (Fig. 2*D*). Importantly, as described later in Figs. 4*A* and 5*C*, when analyzing thymic sections by immunohistochemistry, Aire$^+$ cells were observed only in the medulla and never in the cortex.

Proportion of Aire$^+$ cells per thymus

In the previous experiment, CD45$^+$ cell depletion by AutoMACS leads to the substantial enrichment of mTEC, to the detriment of cTEC. Moreover, only the three last enzymatic digestions were used to generate the enriched population, rendering it more difficult to appreciate the overall proportion of mTEC in a whole thymus. To more accurately assess the proportion of Aire$^+$ cells within the thymus, staining with 5H12 was performed on a whole thymus, after digestion and red cells elimination. By knowing the total number of cells in a thymus, it was easier to estimate the number of both CD45$^-$ cells and MHC-II^{high}, Ly51^{low} CD45$^-$ cells, despite these populations being very rare. Based on these analyses, CD45$^-$ cells represent 1% ± 0.4 of a whole thymus and among these CD45$^-$ cells, the MHC-II^{high} Ly51$^-$ CD45$^-$ subset represent 0.5% ± 0.2 (Fig. 2*E*). CD45$^-$ cell estimation is slightly higher than Gray et al. Indeed, they found CD45$^-$ cells represent ~0.5% of thymic cellularity (13). By extrapolation using the previous results and assuming that there is no alteration of the cell distribution after enrichment within the CD45$^-$ MHC-II^{high} Ly51^{low} population, there are ~7000 CD45$^-$ MHC-II^{high} Ly51^{low} Aire$^+$ cells in a thymus. Thus, Aire$^+$ cells represent 0.005% ± 0.002 of a whole thymus (Fig. 2*E*) and despite being a very rare subset in the thymus, are of great importance in thymic T cell education.

Expression of costimulatory molecules on MHC-II^{high} Ly51^{low} cells

Several reports have renewed interest in the cell biology of negative selection by showing that in mice, mTECs express a broad range of autoantigens dependent on Aire, which leads to deletion of autoreactive T cells. In addition, different studies have provided strong evidence for the action of several costimulatory molecules in negative selection (16–18). Aire$^+$ cells could not be directly stained for their expression of costimulatory molecules because the fixation necessary for intracellular Aire staining destroys the capacity to detect costimulatory molecules. Thus, we used the forward and side scatter properties of Aire$^+$ cells described earlier (Fig. 2, *A* and *C*), to distinguish these cells within the CD45$^-$ MHC-II^{high} Ly51^{low} population for analysis of the expression of a panel of costimulatory molecules (Fig. 3). Approximately 97% of the putative Aire-expressing mTEC expressed the costimulatory molecules CD40, CD80 and PD-L1, previously reported to be expressed by mTECs (19). PD-L2 and CD86 were also expressed on 80 and 70% of these cells, respectively, but ICOS-L expression was not evident.

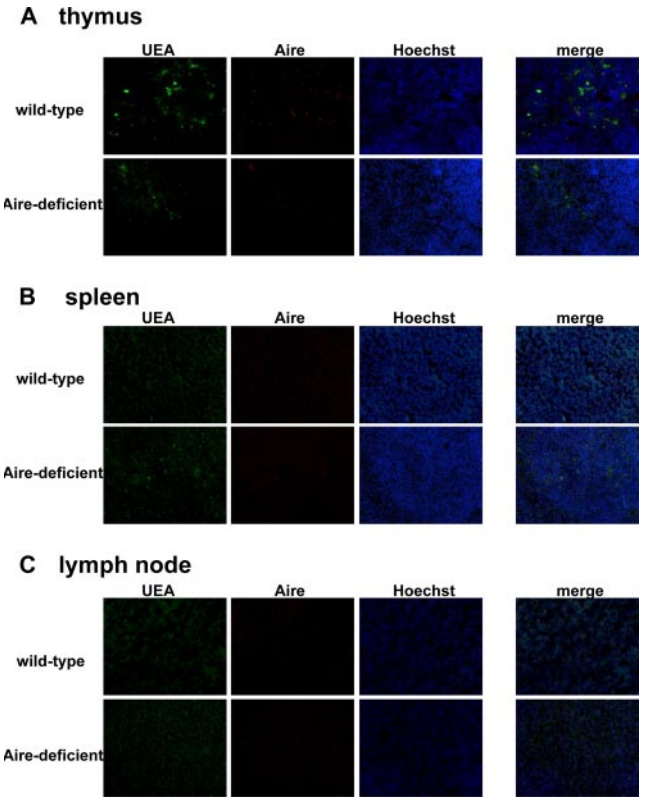

FIGURE 6. Aire immunofluorescence on thymic (*A*), splenic (*B*), and lymph node (*C*) sections in wild-type mice and Aire-deficient mice. Tissues were stained with an anti-Aire Ab (red), an anti-UEA Ab (green) and Hoechst (blue). The immunofluorescence was visualized by fluorescent microscopy at magnification ×20.

Localization of Aire$^+$ cells in tissues

Using our mAb, we examined Aire expression in the thymus, spleen and lymph nodes by immunofluorescence. Aire staining was not detected in the spleen or lymph nodes, but was (Fig. 4*A*) confirmed to be localized to the medulla and at the cortico-medulla junction within the thymus (5, 7). We also found that Aire is located in nuclear dots (Fig. 4, *A* and *B*), as previously shown by immunofluorescence staining with polyclonal Abs on AIRE-transfected cells (10, 20) and confirmed ex vivo in human (21) and mouse (5). Some studies also report a diffuse nuclear staining pattern in a minority of AIRE-transfected cells (10, 20, 22, 23) and ex vivo in human (21), but we did not observe such staining with our monoclonal anti-Aire Ab. Interestingly, when we analyzed Aire$^+$ cells in the thymus by confocal microscopy and focused specifically on single cells, we clearly observed perinuclear, puncta localization inside the nucleus, but excluded from the nucleoli (Fig. 4*B*). This Aire expression pattern suggests localization between the euchromatin and heterochromatin, reinforcing a probable role of Aire or an Aire complex in regulation at the epigenetic level. These results suggest that Aire expression is restricted to the thymic medulla compartment and specifically to thymic epithelial cells.

Aire expression in dendritic cells and UEA$^+$ lymph node cells

Aire plays a role in tolerance and DC are a key element in tolerance induction, thus DC are a potential site for an Aire expression. Indeed, Aire expression in DC has been reported by different teams, but only at the mRNA level. Although we were unable to detect any Aire$^+$ cells in spleen and lymph node by immunohistochemistry, we used nested PCR to examine *Aire* mRNA expression in different DC populations from the thymus and

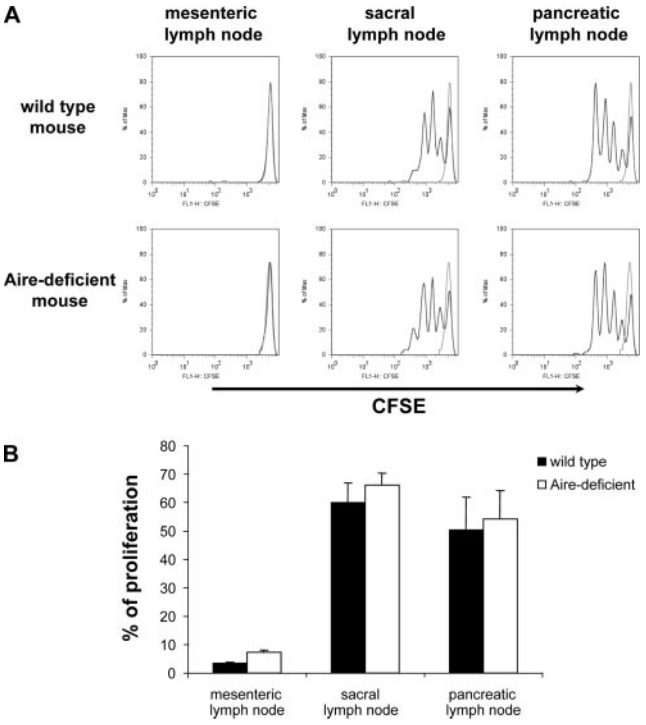

FIGURE 7. OT-I proliferation in peripheral lymph nodes (mesenteric, sacral and pancreatic) of RIP-OVA^{high} Aire$^{+/+}$ ($n = 4$) and RIP-OVA^{high} Aire$^{-/-}$ mice ($n = 4$). *A*, After i.v. transfer of CFSE-labeled OT-I cells into RIP-OVA^{high} transgenic mice wild type or Aire-deficient in C57BL/6 mice, OT-I proliferation was assessed 60 h after transfer in mesenteric, sacral and pancreatic lymph nodes from RIP-OVA^{high} Aire$^{+/+}$ by flow cytometry. *B*, Quantification of data was represented as the percent divided cells among donor OT-I T cells (mean ± SD) for each tissue.

spleen (Fig. 5*A*). This approach detected *Aire* mRNA in all DC subsets and in mTEC. Interestingly, Ruan et al. identified and described 11 different Aire splicing forms resulting from combinations of four alternative splicing units (exon 10, exon 11, 12 bp in exon 6, 13 bp in exon 8). We performed a real time quantitative PCR assay on different regions of the *Aire* mRNA transcript (5′ end [exon 1–2], middle [exon 7–8] and 3′ end [exon 13–14]), which are normally unaffected by splicing, in different thymic and splenic DC populations from WT and Aire$^{-/-}$ mice (Fig. 5*B* and data not shown). The same expression profile was obtained for all three regions of the Aire transcript, with mTEC expressing Aire 100-fold higher than DCs, though DC from wild-type mice always showed higher expression than that seen for in Aire$^{-/-}$ populations (Fig. 5*B*).

To support the view that Aire was poorly expressed by DC, Aire expression was examined in different tissues using β-galactosidase as a surrogate marker, because the targeting vector was inserted the LacZ reporter gene under the control of the endogenous Aire promoter. In Aire$^{+/-}$ mice, β-galactosidase expression was localized to the medulla and at the cortico-medulla junction within the thymus, with no staining in the spleen (Fig. 5*C*), where DC are plentiful. Finally, flow cytometric analysis of DCs from different tissues failed to reveal Aire expression in these cells (Fig. 5*D*).

Recently Lee et al. found Aire mRNA expression in CD45$^-$ UEA$^+$ epithelial cells within the lymph node and implicated Aire in peripheral tolerance (11). After enrichment on CD45$^-$ cells from spleen or lymph node, cells stained for UEA showed no expression of Aire by flow cytometry (Fig. 5*E*). Similarly, no Aire expression was detected on UEA$^+$ cells in spleen or lymph node tissue sections (Fig. 6).

Aire does not play a role in the periphery

Lee et al. reported that a population of lymph node stromal cells endogenously express tissue-specific Ags for peripheral tolerance induction and suggested that this process may be Aire dependent (11). They used the iFABP-tOVA transgenic mouse model in which expression of a truncated, cytosolic form of OVA was targeted to mature intestinal epithelial cells in the small bowel (24). Here, we use a model referred as RIP-OVA^{high}, which is transgenic for a cytosolic form of OVA expressed under Rat Insulin Promoter (RIP) in the thymus and the pancreas (25). To assess a role for Aire, we measured the proliferation of OVA-specific (OT-I) TCR transgenic CD8$^+$ T cells, which recognize a complex of the OVA peptide (amino acids 257–264) with the MHC class I molecule H-2K^b (26), after i.v. transfer of the cells into RIP-OVA^{high} mice either wild-type or deficient for Aire (Fig. 7). As expected OT-I proliferation was detected in sacral and pancreatic lymph nodes of RIP-OVA^{high} Aire$^{+/+}$ mice but not in mesenteric lymph nodes. Importantly, we observed the same level of OT-I cell proliferation in RIP-OVA^{high} Aire$^{-/-}$ mice, arguing against a role for Aire in the presentation of our tissue-specific Ag.

Discussion

In this study, we use a mAb specific for Aire to show that Aire is only found in a rare population of cells from the thymus that are CD45$^-$ Ly51^{low} MHC-II^{high} and EpCam^{high}. These cells are localized to the thymic cortico-medullary junction and in the medulla. This specific localization correlates with their functional role in the negative selection. Moreover, Aire is expressed as dots/puncta in the nucleus probably at the interface between euchromatin and heterochromatin, reinforcing a probable role of Aire or an Aire complex in the regulation at the epigenetic level. Moreover, Aire$^+$ cells were observed to capture low levels of Hoechst, suggesting large-scale chromatin rearrangements consistent with the role of Aire as a global transcriptional regulator.

Interestingly, these cells express a specific repertoire of costimulatory molecules: CD40, CD80 and PD-L1, some of which have been reported to be expressed on mTECs (19). This expression profile reinforces the role of CD40 and CD80 described for negative selection (27, 28). Interestingly, Nishimura et al. have shown that negative selection occurs normally in the PD-1 receptor deficient mice (29), despite its broad expression in both the thymic cortex and medulla (30) and involvement in positive selection (31).

In the absence of CD80/CD86 costimulation, T cells express lower levels of ICOS, suggesting cross-regulation of costimulatory pathways (32). Thymic expression of ICOS is primarily restricted to the medulla and the cortico-medullary junction (33) and this localized expression suggests that ICOS may have a role in thymic selection. As shown in Fig. 3, ICOS-L was poorly expressed by mTEC, but this does not eliminate the potential involvement of the ICOS pathway in negative selection. Indeed, DCs have been shown to be involved in negative selection (34) and may express ICOS-L on their surface.

Interestingly, a terminal differentiation model of mTECs has been proposed in which tissue-specific Ag expression is induced as a result of de-repressed genes and Aire expression during mTEC maturation (35). The authors assumed mTEC maturation coincided with the increase of MHC-II and CD80 molecules. Results obtained here show that nearly 100% of CD45$^-$ MHC-II^{high} Ly51^{low} cells express Aire and CD80 supporting the terminal differentiation model.

Because APECED is an autoimmune disorder, immunologically relevant organs might be expected to express Aire. In this study, we focused on immunology organs. Staining for Aire in thymus,

spleen and lymph node, suggests that Aire expression is restricted to the thymic medulla compartment and specifically to thymic epithelial cells. The expression pattern of human AIRE was detected at mRNA level in the thymus, lymph node and fetal liver. AIRE expression was also found in the spleen and tissues affected in APS1, such as the adrenal cortex and pancreas by some groups (36) but not by others (2). Analysis by immunofluorescence and immunohistochemistry with a polyclonal anti-AIRE Ab revealed that human AIRE protein expression was also seen in thymic medulla, lymph node, spleen and fetal liver, but not observed in any other tissues (7, 20). At the thymic level, these studies revealed that AIRE expression is predominantly detected in thymic medulla (20), at the margins of Hassall's corpuscles (37) and in scattered cells in the cortex (21). Reports of the tissue distribution of murine Aire mRNA are also discordant. Although it could not be detected by Northern blot analysis, quantitative PCR revealed the presence of Aire in the heart (8, 38, 39), lung, testis (8, 39), thymus (39), and lymph node (7, 10). However, there are conflicting reports of Aire mRNA expression in the spleen (7, 8, 39), kidney, brain (8, 10), and liver (39) (10). Furthermore, a single report found Aire mRNA in the ovary, adrenal gland and thyroid (39). At a protein level, Halonen et al. used a polyclonal Ab, to detect the presence of Aire in the thymus, spleen, lymph nodes and bone marrow, in different cell populations of lymphoid and myeloid lineages (lymphocytes, monocytes, and polynuclear leukocytes) and in addition, in the reticular epithelial cells and corpuscle cells of the thymus and epithelial cells of the lymph vessels (10). Outside of the immune system, Aire protein was detected in the brain, liver, kidney, pancreas, intestinal canal, gonads, pituitary, thyroid, and adrenal glands. The cells expressing Aire represent diverse cell types, including epithelial cells, neurons and glial cells. Using a different polyclonal Ab, Adamson et al. showed the presence of Aire protein in immunologically relevant tissues (in the thymic medulla, in B- and T cell areas of the lymph nodes, in the spleen in T cells areas of the white pulp) and a restricted number of peripheral tissues (epithelial cells of the lung, kidney, neurons within cortex and hippocampus, in the ovary, testis and in the goblet cells of the gut) (40). They found a more restricted and slightly different pattern of Aire expression compared with Halonen et al. (10), but both of these studies used polyclonal Abs. Indeed mAb has an inherent monospecificity toward a single epitope that allows fine detection and quantitation of small differences in Ag.

Interestingly, Aire mRNA expression in DCs, whether WT or Aire$^{-/-}$, was always less than that seen in Aire$^{-/-}$ thymic CD45^{-} cells, revealing an extremely low (perhaps irrelevant) level of expression in DCs. The level of Aire expression observed in CD45^{-} thymic cells of Aire-deficient mice may come from a possible contamination of genomic DNA or from aberrant splicing. Finally, we decided to analyze Aire protein expression in DCs from different tissues: thymus, spleen and lymph nodes, by flow cytometry. Indeed, 5H12 Ab has been generated against amino acid encoded by exon 14, which has been described to be present in all Aire isoforms. Thus, we expected to be able to detect all Aire isoforms. These results suggested the presence of residual Aire mRNA in DCs, the level of which is too low to detect protein expression by flow cytometry. Recently Lee et al. found Aire mRNA expression in CD45^{-} UEA^{+} epithelial cells within the lymph node and suggest an important role of Aire in peripheral induction of the tolerance (11). However, when we stained these cells for Aire expression, no Aire-positive signal was detected.

Considering these observations, it is possible, although unlikely, that Aire is present in DCs and/or lymph nodes epithelial cells in very small amounts that are undetectable by flow cytometry, but may still be playing a potent and important role in these cells. Alternatively, a certain level of mRNA may be necessary to induce translation of Aire transcript into protein and this critical amount is absent from DCs. Another possibility is that these cells are only capable of inducing Aire expression under specific conditions to participate in peripheral tolerance.

This is the first report describing analysis of these cells using flow cytometry and this technique opens the door for more thorough analysis of this unique Aire^{+} population of TECs.

Acknowledgments

We are grateful to Viki Milovac for cell sorting.

Disclosures

The authors have no financial conflict of interest.

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