Short Communication

Human Peripheral Lymphoid Tissues Contain Autoimmune Regulator-Expressing Dendritic Cells

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Autoimmune regulator (AIRE) modulates the expression of tissue-restricted antigens (TSAs) and promotes central tolerance in the thymus. However, few autoreactive T cells escape negative selection and reach the periphery, where peripheral tolerance is required to avoid autoimmunity. Murine lymph nodes (LNs) have been shown to contain "stromal" cells expressing AIRE and TSAs. Here we report the occurrence of AIRE-expressing cells in human peripheral lymphoid tissues, including LNs, tonsils, and gut-associated lymphoid tissue, with the exception of the spleen. Notably, AIRE^{+} cells are absent in fetal LNs and, in postnatal life, they are more numerous in abdominal than in superficial LNs, thus suggesting that their development in periphery may depend on instructive signals from microenvironment and antigen challenge. Extrathymic AIRE^{+} cells show a dendritic morphology, consistently express human leukocyte antigen-DR (HLADR) and fascin, and are largely positive for CD11c and S100 and for the dendritic cell-activation markers CD40, CD83, DC-LAMP/CD208, and CCR7. Lymphoid, myelomonocytic, mesenchymal, and epithelial cell lineage markers are negative. The HLADR[high]/AIRE[+] cell fraction isolated from mesenteric LNs expressed TSAs (insulin, CYP17A1, and CYP21A2), as well as molecules associated with tolerogenic functions, such as interleukin-10 and indoleamine 2,3-dioxygenase. Data indicate that AIRE^{+} cells in human peripheral lymphoid tissues correspond to a subset of activated interdigitating dendritic cells expressing TSAs and the tolerogenic molecules indoleamine 2,3-dioxygenase and interleukin-10, suggestive of a potential tolerogenic function. *(Am J Pathol 2010, 176:1104–1112; DOI: 10.2353/ajpath.2010.090956)*

Central tolerance in the thymus is predominantly mediated by a subset of medullary epithelial cells[1] that have a distinctive phenotype (cytokeratin-5[+], claudin-4[+], *Ulex europaeus* agglutinin [UEA]-1[+], and major histocompatability complex [MHC]-II[high])[2] and express autoimmune regulator (AIRE), a transcription factor that regulates the expression of peripheral tissue-specific antigens.[3–5] Although most newly generated autoreactive T cells are negatively selected on interaction with AIRE[+] medullary epithelial cells, a few escape central tolerance selection and reach the periphery, where peripheral tolerance induction is then required to prevent autoimmunity.[6] It has been postulated that AIRE may also play a role in peripheral tolerance induction,[5] but AIRE expression in human extrathymic tissues remains controversial. In particular, AIRE mRNA has been detected in various peripheral lymphoid and nonlymphoid tissues and is barely detectable in peripheral blood mononuclear cells[7–9]; however, expression of AIRE protein outside of thymus has been not firmly demonstrated.[10–12]

Recently, two independent studies reported that CD45^{−}MHC-II^{high} cells expressing AIRE are present in murine lymph nodes (LNs).[13,14] Gene expression analysis showed that these cells express a subset of tissue-specific antigens that only partially overlap with those

found in medullary epithelial cells and induce deletion of autoreactive T cells.

We have performed an extensive analysis of AIRE expression in human tissues and found that, in addition to the thymus, AIRE^{+} cells regularly occur in peripheral lymphoid tissues. Phenotypic analysis of these cells indicates that they correspond to a subset of activated interdigitating dendritic cells expressing molecules associated with tolerogenic functions, such as indoleamine 2,3-dioxygenase and interleukin-10.

Materials and Methods

Tissues

A wide range of normal human formalin-fixed paraffin-embedded and freshly frozen samples were retrieved from the archive of the Department of Pathology, Spedali Civili of Brescia, in accordance with the protocols of the Spedali Civili of Brescia ethical board (Table 1). Fetal tissues at different gestational ages were obtained from spontaneous abortions (first trimester) and from six autopsies (second and third trimester). From all tissues at least three different samples were examined. Postnatal tissues included tonsils (20) and reactive LNs (95) from different anatomical sites (27 cervical, 25 mesenteric, 15 axillary, 12 inguinal, 8 peripancreatic, and 8 pulmonary LNs) and from patients of different ages (17, 34, and 44 LNs, respectively, from patients aged <30, 31 to 60, and >60 years) and gender (53 males and 42 females). Normal thymic biopsy samples, obtained anonymously from patients who underwent elective surgery for correction of cardiovascular defects, were used as positive controls for AIRE expression, whereas negative controls were represented by the omission of the primary antibody as well as LNs and thymic biopsy samples from patients with severe human primary immunodeficiencies lacking AIRE expression.[15]

Immunohistochemical Procedures

Sections from both paraffin-embedded and frozen tissue blocks were stained using a mouse monoclonal anti-human AIRE antibody.[12] Cryostat sections were fixed in acetone for 10 minutes before staining, whereas paraffin sections underwent heat-based treatment for antigen retrieval using a microwave oven or a thermostatic bath in 1.0 mmol/L EDTA buffer (pH 8.0). Single immunostains were revealed by the ChemMATE EnVision HRP system (DAKO, Glostrup, Denmark) or the Super-Sensitive Detection System (BioGenex, San Ramon, CA), followed by diaminobenzidine/hydrogen peroxide as chromogen and hematoxylin as counterstain. Double immunostains were performed for AIRE and the antigens listed in Table 2, for which appropriate biotinylated secondary antibodies were applied (DAKO), followed by streptavidin-conjugated alkaline phosphatase (DAKO); the chromogen reaction was developed by either Ferangi Blue or Vulcan Red Chromogen Kits (Biocare Medical, Concord, CA), and nuclei were counterstained with methyl green.

Table 1. AIRE Expression in Human Fetal and Adult Tissues

Tissues	Results
Cardiovascular system	
Heart, blood vessels	Negative
Endocrine glands	
Thyroid, parathyroid, adrenal	Negative
Eye and annexa	
Retina, cornea, conjunctiva, lacrimal glands	Negative
Female genital system	Negative
Ovary and uterine tubes, vulva, cervix and uterus	Negative
Placenta	Negative
Gastrointestinal tract	
Tongue, lips, oral mucosae, esophagus, stomach	Negative
Small and large bowel	Rare positive cells in GALT (*negative)
Appendix	Rare positive cells in GALT (*negative)
Liver, bile ducts and gallbladder	Negative
Pancreas	Negative
Hemolymphopoietic system	
Bone marrow	Negative
Thymus	Positive
Lymph nodes, tonsils and adenoids	Positive (*negative)
Spleen	Negative
Integumentary system	
Skin and skin annexa	Negative
Male genital system	
Testis and epididymis, seminal vesicles, prostate	Negative
Mammary gland	Negative
Nervous system	
Central and peripheral nervous system	Negative
Respiratory system	
Nasal cavity, larynx, trachea, bronchi, lung, pleura	Negative
Salivary glands	
Major and minor salivary glands	Negative
Soft tissues	
Muscle, nerve, bone, cartilage	Negative
Urinary system	
Kidney, urinary tract and bladder	Negative

When not specified, fetal and postnatal tissues show identical expression.

GALT, gut-associated lymphoid tissues, including Peyer's patches.

*Different expression in fetal tissues compared to adult.

Flow Cytometry Analysis (Fluorescence-Activated Cell Sorting) and Immunomagnetic Cell Depletion

Cell suspensions were obtained by mechanical disruption in RPMI 1640 medium supplemented with 10% fetal calf serum from mesenteric LNs draining inflammatory intestinal diseases (eg, diverticulitis or intestinal occlusion). After red blood cell lysis, cells were resuspended in EDTA/fluorescence-activated cell sorting (FACS) buffer (2% fetal calf serum, 0.02% NaN_3, and 5 mmol/L EDTA in PBS) and passed through a 100-μm filter. FACS analysis was performed either on cell suspension from whole LNs or after immunomagnetic cell depletion using CD45-conjugated microbeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). For super-

Table 2. List of the Antibodies Used in Double Immunostains to Characterize AIRE-Expressing Cells in Human Lymph Nodes and Corresponding Results

Antibodies	Species/isotype/clone/dilution/source	Results
Lymphoid antigens		
CD3	Rp/n.d./SP7/1:100/LV	Negative
CD5	Mm/IgG1/4C7/1:50/NC	Negative
CD20	Mm/IgG2a/L26/1:250/DK	Negative
Myeloid and macrophage antigens		
CD14	Mm/IgG2a/7/1:50/NC	Negative
CD33	Mm/IgG2b/PWS44/1:100/NC	Negative
CD68	Mm/IgG1/KP1/1:300/DK	Negative
CD163	Mm/IgG1/10D6/1:50/TS	Negative
DC-associated antigens		
Myeloid DCs		
CD1a	Mm/IgG1/010/1:50/DK	Negative
CD1c/BDCA-1*	Mm/IgG2a/AD5-8E7/1:50/MC	Negative
CD11c	Mm/IgG2a/5D11/1:30/NC	Positive (91%)[†]
CD207/langerin	Mm/IgG2b/12D6/1:200/VL	Negative
CD209/DC-SIGN*	Mm/IgG2b/n.d./[‡]	Negative
Factor XIIIa	Rp/n.d./n.d./1:100/BG	Negative
Fascin	Mm/IgG1/55K-2/1:50/DK	Positive (100%)
S-100	Rp/n.d./n.d./1:5000/DK	Positive (78%)
Plasmacytoid DCs		
CD123	Mm/IgG2a/7G3/1:150/PH	Negative
CD303/BDCA-2	Mm/IgG1/124B3.13/1:50/DC	Negative
CD2AP	Mm/IgG1/B-4/1:1000/SC	Negative
Activation and maturation DC-associated antigens		
CCR7	Mm/IgG2a/150503/1:50/RD	Positive (74%)
CD208/DC-LAMP	Mm/IgG1/104.G4/1:100/IM	Positive (83%)
CD40	Mm/IgG2b/11E9/1:20/NC	Positive (100%)
CD83	Mm/IgG1/HB15e/1:20/PH	Positive (42%)
CD86*	Gp/n.d./n.d./1:20/SC	Negative
HLADR	Mm/IgG1/CR3-43/1:150/DK	Positive (100%)
Stromal and endothelial antigens		
Desmin	mm/IgG1/33/1:100/BG	Negative
Podoplanin	Mm/IgG1/D2-40/1:40/SG	Negative
Smooth muscle actin	Mm/IgG1/HHF35/1:100/DK	Negative
Vimentin	Mm/IgG1/V-9/1:100/BG	Negative
CD31	mm/IgG2b/4C9/1:50/NC	Negative
Factor VIII-related antigen	Rp/n.d./n.d./1:100/NK	Negative
Epithelial antigens		
Wide spectrum cytokeratins	Mm/IgG1/MNF116/1:100/DK	Negative
Cytokeratins 8/18/19	Mm/IgG2a/CAM5.2/1:50/BD	Negative
Cytokeratin 5	Rp/n.d./AF138/1:100/CV	Negative
Cytokeratin 8	Rm/n.d./TROMA-1/1:200/[‡]	Negative
Claudin-4	Mm/IgG1/3E2C1/1:100/ZY	Negative
Epithelium-specific antigen	Mm/IgG1/VU-1D9/r.t.d./NC	Negative
UEA-1	Lectin/n.d./n.d./1:600/VL	Negative
Miscellaneous		
CD16	Mm/IgG2a/2H7/1:80/NC	Negative
CD25	Mm/IgG2b/4C9/1:150/NC	Negative
CD34	Mm/IgG1/QBEnd-10/1:200/NK	Negative
CD43	Mm/IgG1/MT1/1:40/NC	Negative
CD45	Mm/IgG1/RP2-18;RP2-22/1:200/NC	Negative
CD45RA	Mm/IgG1/MB1/1:100/BT	Negative
CD45RB	Mm/IgG1/PD7-26/1:200/DK	Negative
CD45RC	Mm/IgG1/MT2/1:5/BT	Negative
CD45RO	Mm/IgG2a/UCL1/1:300/NK	Negative
CLUSTERIN	Mm/IgG1/7D1/1:200/NC	Negative
CXCL12/SDF-1	Mm/IgG1/78018/1:50/RD	Negative
CXXL13/BCA1	Gp/n.d./n.d./1:30/RD	Negative
IDO	Rp/n.d./n.d./1:1500/CM	Positive (92%)

Mn, mouse monoclonal; Rm, rat monoclonal; Rp, rabbit polyclonal; Gp, goat polyclonal; n.d., non definable; r.t.d., ready to use; LV, LabVision Corporation (Fremont, CA); NC, Novocasta (Castle upon Tyne, UK); DK, DAKO (Glostrup, Denmark); TS, Thermo Fisher Scientific (Fremont, CA); MC, MACS (Gladbach, Germany); VL, Vector Laboratories (Burlingame, CA); BG, Biogenex (San Ramon, CA); PH, Pharmingen (San Diego, CA); DC, Dendritics (Lyon, France); SC, Santa Cruz Technology, Inc. (Santa Cruz, CA); RD, R&D Systems (Minneapolis, MN); IM, Immunotech (Praha, Czech Republic); SG, Signet (Dedham, MA); NK, Neomarkers (Fremont, CA); BD, Becton Dickinson (San Jose, CA); CV, Covance (Princeton, NJ); ZY, Zymed (San Francisco CA); BT, Biotest (Dreieich, Germany); CM, Chemicon (Hofheim, Germany).
*Antibodies were applied on frozen sections.
[†]Percentages of double-positive cells/total AIRE^{+} cells.
[‡]Anti-DC-SIGN was kindly provided by M. Cella (Washington University, St. Louis, MO) and anti-cytokeratin 8 by U.H. von Andrian (Harvard Medical School, Boston, MA).

Table 3. Primer Sequences Used for Real Time RT-PCR Analysis

Gene	Forward sequence	Reverse sequence
ACTb	5′-CTGGAACGGTGAAGGTGACA-3′	5′-CGGCCACATTGTGAACTTTG-3′
AIRE	5′-GAGAGTGCTGAGAAGGACA-3′	5′-GTTTAATTTCCAGGCACATGA-3′
IL10	5′-TTCCCAGGCAACCTGCCTAA-3′	5′-TGTCCAGCTGATCCTTCATTTGA-3′
IDO	5′-CCAAAGCAGCGTCTTTCAGTG-3′	5′-GGTGGCATATATCTTCTCATGTCC-3′
CYP17A1	5′-GTAACCGTCTCCTCCTGCTG-3′	5′-ACTTCTGTGCCCTTGTCCAC-3′
CYP21A2	5′-AGAGGGTGTTTGCTGTGGTC-3′	5′-GTGGAGGGACATGATGGACTAC-3′
INSULIN	5′-GCAGCCTTTGTGAACCAACA-3′	5′-GTGTGTAGAAGAAGCCTCGTTCC-3′

ficial staining at least 10^6 cells in 150 μl of EDTA/FACS buffer were incubated with purified mouse anti-human CD40 followed by a goat anti-mouse Alexa 488-conjugated secondary antibody and after staining with CD45-allophycocyanin, human leukocyte antigen-DR (HLADR)-PerCp CD83-phycoerythrin-conjugated antibodies (BD Pharmingen, San Diego CA). At least 500,000 events were acquired by FACSCanto II and analyzed with BD FACSDiva software (version 5.0.1).

Isolation of an AIRE-Enriched Cell Fraction

Cell suspensions from mesenteric LNs have been highly enriched for the AIRE-expressing cell population by combining low-density (OptiPrep) cell separation for dendritic cells followed by immunomagnetic cell sorting using AutoMACS. In brief, cell suspensions from LNs were obtained by mechanical disruption and digestion in a PBS buffer containing 2.5 ml of collagenase/dispase (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNase (1.5 μg/ml). Cells were then resuspended in RPMI 1640 and filtered through a 100-μm cell strainer before isolation by low-density fraction using the OptiPrep protocol C21 (low-density fraction contains 25×10^6 cells). Cells were then washed in MACS buffer before immunomagnetic cell depletion with CD14-, CD19-, and DC-SIGN-conjugated microbeads, according to the manufacturer's instructions (Miltenyi Biotec) (cell fraction contains 7×10^6 cells). The negative fraction was further enriched by positive selection of the HLADR-positive cells using HLADR-conjugated microbeads.

mRNA Extraction and Real-Time PCR

Total mRNAs were extracted from fresh frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. AIRE and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were measured using "assay on demand" real-time PCR kits (AIRE: Hs00230833_m1; GAPDH: Hs99999905_m1; Applied Biosystems). cDNA was combined with 10 μl of TaqMan Universal PCR Master Mix and 2 μl of primers/probe in the thermal cycling conditions as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute. Samples were analyzed using the MJ Research PTC-200 Peltier Thermal Cycler and MJ Opticon Monitor analysis software 3.1 (Bio-Rad Labora-

tories, Hercules, CA). The expression level of the target gene was achieved by using the comparative cycle of threshold (C_t) method, normalized to the housekeeping gene GAPDH and represented as arbitrary units. mRNA from AIRE-enriched cell fractions was extracted as described. Total mRNA was reverse-transcribed to cDNA using SuperScript III (Invitrogen), 10 mmol/L dNTP mix, oligo(dT)18 primer, and RiboLock RNase inhibitor (Fermentas, Vilnius, Lithuania), and cDNA was run using an ABI Prism 7900 sequence Detection System (Applied Biosystems) and quantitative PCR SYBR Green Core Kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. SYBR Green fluorescence was measured after each extension step, and specificity of amplification was evaluated by melting curve analysis. Relative gene expression levels were calculated using the comparative C_t method. Every sample was run in three parallel reactions. Primers for all target genes and housekeeping β-actin (*ACTb*) gene are listed in Table 3.

Image Elaboration and Cell Counting

Microscopic images were acquired with an Olympus DP70 digital camera mounted on an Olympus Bx60 microscope using Cell^F imaging software (Soft Imaging System GmbH, Münster, Germany). AIRE$^+$ cells were counted on 10 different high-power fields (corresponding to 8 mm^2 of tissue) for each section, selecting areas with the highest number of positive cells; values are expressed as number of cells/mm^2 $\pm$ SD. Likewise, the number of AIRE$^+$ cells coexpressing CD11c, S-100, fascin, HLADR, CD40, CD83, CD208/DC-LAMP, CCR7, and indoleamine 2,3-dioxygenase (IDO) were evaluated in 10 high-power fields, and positive cells were expressed as a percentage of double-positive cells/total AIRE$^+$ cells.
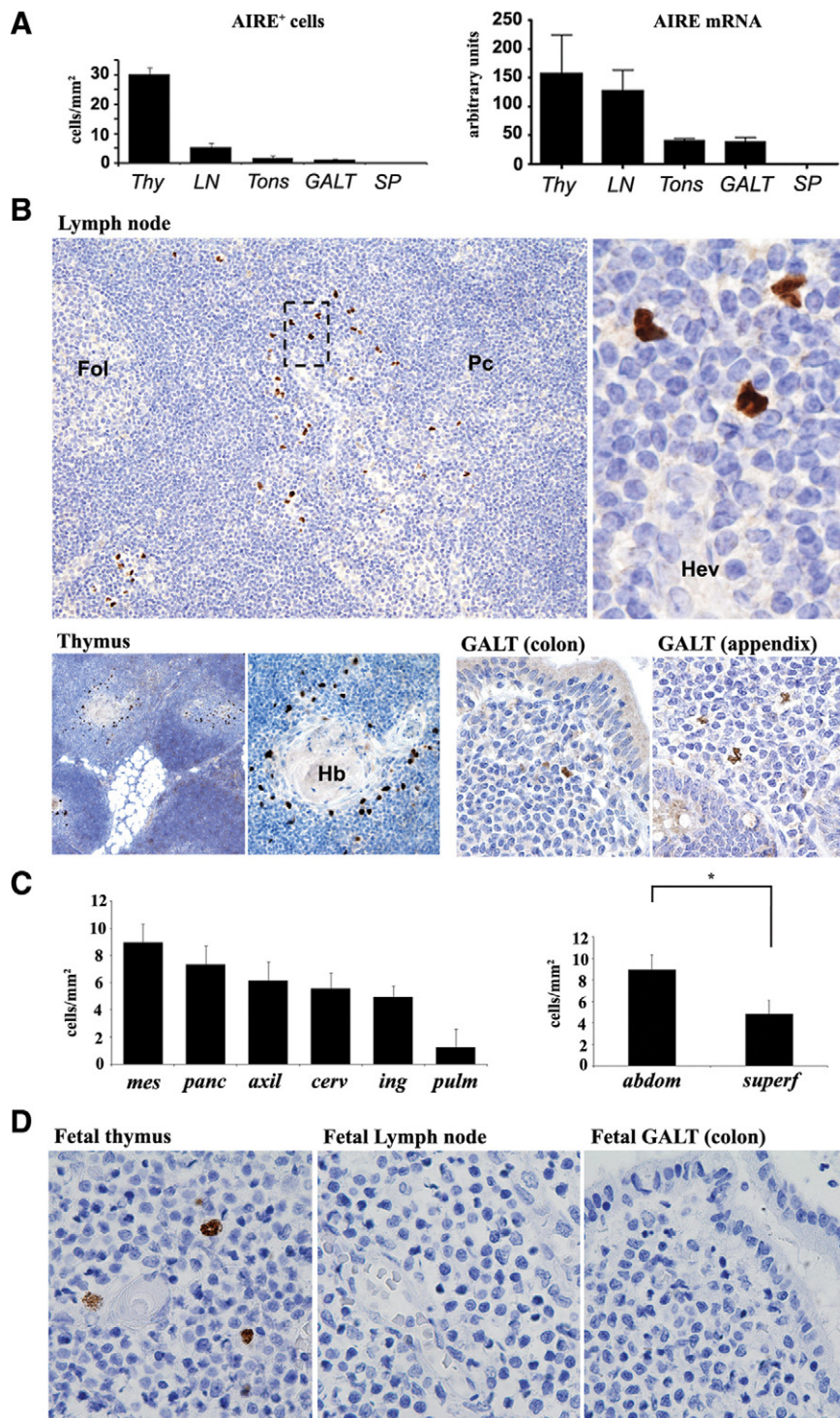
Statistical Analysis

Statistical analysis was performed using the Mann-Whitney test for unpaired nonparametric data. Differences were considered significant when $P < 0.05$.

Results

Extrathymic Expression of AIRE Is Restricted to Peripheral Lymphoid Tissues

Using immunohistochemistry we investigated the expression of AIRE in a large series of human adult and fetal

A

AIRE⁺ cells

AIRE mRNA

B

Lymph node

Fol

Pc

Hev

Thymus

Hb

GALT (colon)   **GALT (appendix)**

C

D

Fetal thymus   **Fetal Lymph node**   **Fetal GALT (colon)**

Figure 1. Expression of AIRE in human thymus and peripheral lymphoid tissues. **A:** The number of AIRE⁺ cells/mm² evaluated by immunohistochemistry (**left plot**) and AIRE mRNA levels analyzed by quantitative RT-PCR (**right plot**) show that extrathymic AIRE expression occurs particularly in LNs, albeit at lower level than in the thymus (Thy), tonsils (Tons), and GALT but not in the spleen (SP). **B:** In LNs (**top panel**) AIRE⁺ cells are distributed as scattered cells in the interfollicular area/outer paracortex (Fol, follicle; Pc, paracortex), in the vicinity of high endothelial venules (Hev), as indicated in the higher magnification from the **dotted box** area (original magnification: ×10 and ×80). In the thymus (**bottom panel**, **left**), AIRE⁺ cells occur in the medulla and correspond to medullary epithelial cells around Hassall's bodies (Hb) (original magnification: ×4 and ×10). Rare cells are present in the colon, appendix, and GALT (**bottom panel**, **right**) (original magnification: ×20). **C:** The number of nodal AIRE⁺ cells differs in anatomical regions (**left plot**). The difference between AIRE⁺ cells in abdominal (mesenteric plus peripancreatic) compared with superficial (axillary, inguinal, and cervical) LNs is statistically significant (**right plot**; *$P < 0.001$). mes, mesenteric; panc, peripancreatic; axil, axillary; cerv, cervical; ing, inguinal; pulm, pulmonary; abdom, abdominal; superf, superficial. **D:** In the fetus, only the thymus contains AIRE⁺ cells in the medulla, whereas no positive cells occur in LNs and GALT (all panels, original magnification: ×40). All immunostains for AIRE were performed using diaminobenzidine/hydrogen peroxide as the chromogen and hematoxylin as the nuclear counterstain. Bar graphs show averages and SEs of at least three independent measurements.

tissues (Table 1). In adult samples AIRE-expressing cells were identified in medullary epithelial cells of the thymus and in peripheral lymphoid tissues including LNs, tonsils, and gut-associated lymphoid tissue (GALT), with the notable exception of the spleen (Figure 1, A and B). Extrathymic AIRE⁺ cells were regularly found in the LNs, albeit at lower numbers than in the thymus (5.43 ± 1.27 versus 30.29 ± 4.79 AIRE⁺ cells/mm²); only occasional AIRE⁺ cells were detected in the tonsils and GALT (1.8 ± 0.6

and 1.1 ± 0.2 AIRE⁺ cells/mm², respectively). Immunohistochemical data were confirmed by quantitative RT-PCR analysis (Figure 1A). In fetal tissues AIRE⁺ cells were detectable only in the thymus at all gestational ages (first, second, and third trimesters) (Figure 1D) and were more numerous in full-term fetuses (data not shown).

In the LNs AIRE⁺ cells showed irregularly shaped nuclei and were located in the outer paracortex/interfollicular area, close to high endothelial venules (Figure 1B).

Their distribution differed in various anatomical sites, with the highest number occurring in mesenteric (8.9 ± 1.25 AIRE$^+$ cells/mm^2) and peripancreatic (7.3 ± 1.22 AIRE$^+$ cells/mm^2) LNs compared with axillary (6.4 ± 1.17 AIRE$^+$ cells/mm^2), cervical (5.6 ± 1.42 AIRE$^+$ cells/mm^2), inguinal (4.9 ± 1.16 AIRE$^+$ cells/mm^2), and pulmonary (1.2 ± 0.83 AIRE$^+$ cells/mm^2) LNs. The difference between the number of AIRE$^+$ cells found in abdominal versus superficial LNs was statistically significant (6.70 ± 2.2 vs. 4.5 ± 3.0 AIRE$^+$ cells/mm^2; $P < 0.001$) (Figure 1C). Interestingly, the number of AIRE$^+$ cells increased during adult age, peaked between 31 and 60 years, and lowered in elderly individuals (<30 years: 4.1 ± 4.3 AIRE$^+$ cells/mm^2; 30–60 years: 5.5 ± 2.74 AIRE$^+$ cells/mm^2; >60 years: 4.7 ± 3.01 AIRE$^+$ cells/mm^2), but the differences did not reach statistical significance. AIRE$^+$ cells were similarly distributed between genders (data not shown).

AIRE-Expressing Cells Show a Myeloid Dendritic Cell Phenotype

With the aim of characterizing the nature of the AIRE$^+$ cells occurring in LNs, we performed a series of double immunostains (Table 2, Figure 2A), which showed that AIRE$^+$ cells consistently coexpressed HLADR and fascin and were frequently positive for the myeloid dendritic cell markers CD11c and S100 (91.2 ± 1.6 and 78.9 ± 1.4% of total AIRE$^+$ cells, respectively). In addition AIRE$^+$ cells strongly expressed antigens typically found on activated or mature dendritic cells, such as CD40, CD208/DC-LAMP, CCR7, and CD83 (respectively, 90.4 ± 1.35, 83.2 ± 1.28, 74.3 ± 1.72, and 42.8 ± 1.86% of total AIRE$^+$ cells). Interestingly, all double-positive immunostains showed that the AIRE$^+$ cells displayed an obvious dendritic morphology and were admixed with AIRE$^-$ cells showing similar features and phenotype. Notably, AIRE$^+$ cells were negative for CD45 common epitopes and isoforms (RA, RB, RC, and RO). It should be noted that lack of CD45 antigens is not in contrast with the dendritic cell nature of the AIRE$^+$ cells, because we found that interdigitating dendritic cells occurring in dermatopathic lymphadenitis were largely negative for CD45 (Figure 2A). The immunohistochemical data have been confirmed on cell suspensions obtained by low-density gradient enrichment from mesenteric LNs, in which the CD45^{low}HLADR^{high} cell fraction showed high levels of CD40 and a light increase in CD83 density (Figure 2B). Because data obtained in mice showed that nodal AIRE$^+$ cells probably represent fibroblastic reticulum cells, we used a series of antibodies identifying this cell population of the nodal stroma (such as smooth muscle actin, vimentin, desmin, UEA-I, and subsets of cytokeratins), all showing negative results on AIRE-expressing cells (Figure 2A). Taken together, these data indicate that nodal AIRE$^+$ cells represent a subset of interdigitating dendritic cells with a mature/activated phenotype.

AIRE-Expressing Dendritic Cells Show a Tolerogenic Phenotype

In keeping with data obtained in mice,[13,14] flow cytometry showed that the CD45$^{low/-}$/HLADR^{high} cells represent a minor fraction (0.2 to 0.4%) of the total LN cells (data not shown). This observation, along with the lack of a specific surface cell marker for the AIRE$^+$ cells, made cell sorting of this cell population virtually impossible. We therefore investigated the phenotype and potential biological role of the AIRE$^+$ cells, performing *in vitro* studies on cell suspensions highly enriched for AIRE-expressing cells obtained from human mesenteric LNs, by using a low-density gradient followed by negative selection of monocytes (CD14), B-cells (CD19), and immature dendritic cells (DC-SIGN/CD209), and positive selection for HLADR. Notably, only the enriched HLADR$^+$CD14$^-$CD19$^-$CD209$^-$ cell fraction but not the HLADR$^-$CD14$^-$CD19$^-$CD209$^-$ cell fraction showed expression of AIRE mRNA and the AIRE-dependent tissue-restricted antigens insulin, CYP17A1, and CYP21A2. AIRE and tissue antigen mRNA levels from LN sorted cells were lower than those obtained from CD45-depleted thymic stromal cells (Figure 3).

To evaluate the hypothesis that AIRE$^+$ dendritic cells of human LNs may express molecules related to a tolerogenic phenotype, using the same cell enrichment procedure, we analyzed by real-time PCR mRNA levels of IDO and interleukin (IL-10) and found that they were selectively expressed in the HLADR$^+$CD14$^-$CD19$^-$CD209$^-$ cell fraction, whereas they were absent in the HLADR$^-$CD14$^-$CD19$^-$CD209$^-$ nodal cell fraction, as well as in CD45-depleted thymic stromal cells. Double immunohistochemical analysis confirmed that the vast majority of AIRE$^+$ cells express IDO (92.5 ± 3.8% IDO$^+$ cells of total AIRE$^+$ cells) (Figure 3).

Discussion

We investigated the expression of AIRE in a large series of extrathymic human fetal and postnatal tissues and found that rare cells exclusively occurring in postnatal peripheral lymphoid tissues (with the notable exception of the spleen) contain AIRE protein and mRNA. Nodal AIRE$^+$ cells were represented by scattered cells located in the outer paracortex/interfollicular area, close to high endothelial venules, a crucial area for homing and cellular traffic of immune cells.[16,17] Interestingly, AIRE$^+$ cells were more numerous in abdominal and particularly in mesenteric LNs, anatomical sites highly exposed to antigens. In human fetuses AIRE$^+$ cells were detectable only in the thymus, whereas they were not found in LNs and GALT. These observations indicate that AIRE expression is differently regulated in the thymus and in peripheral lymphoid tissues and that development of AIRE$^+$ cells in the periphery may depend on instructive signals from the microenvironment.

Recently, two studies showed that murine LNs contain AIRE$^+$ stromal cells that express MHC-II antigens. The non-hemopoietic, stromal nature of these AIRE$^+$ cells
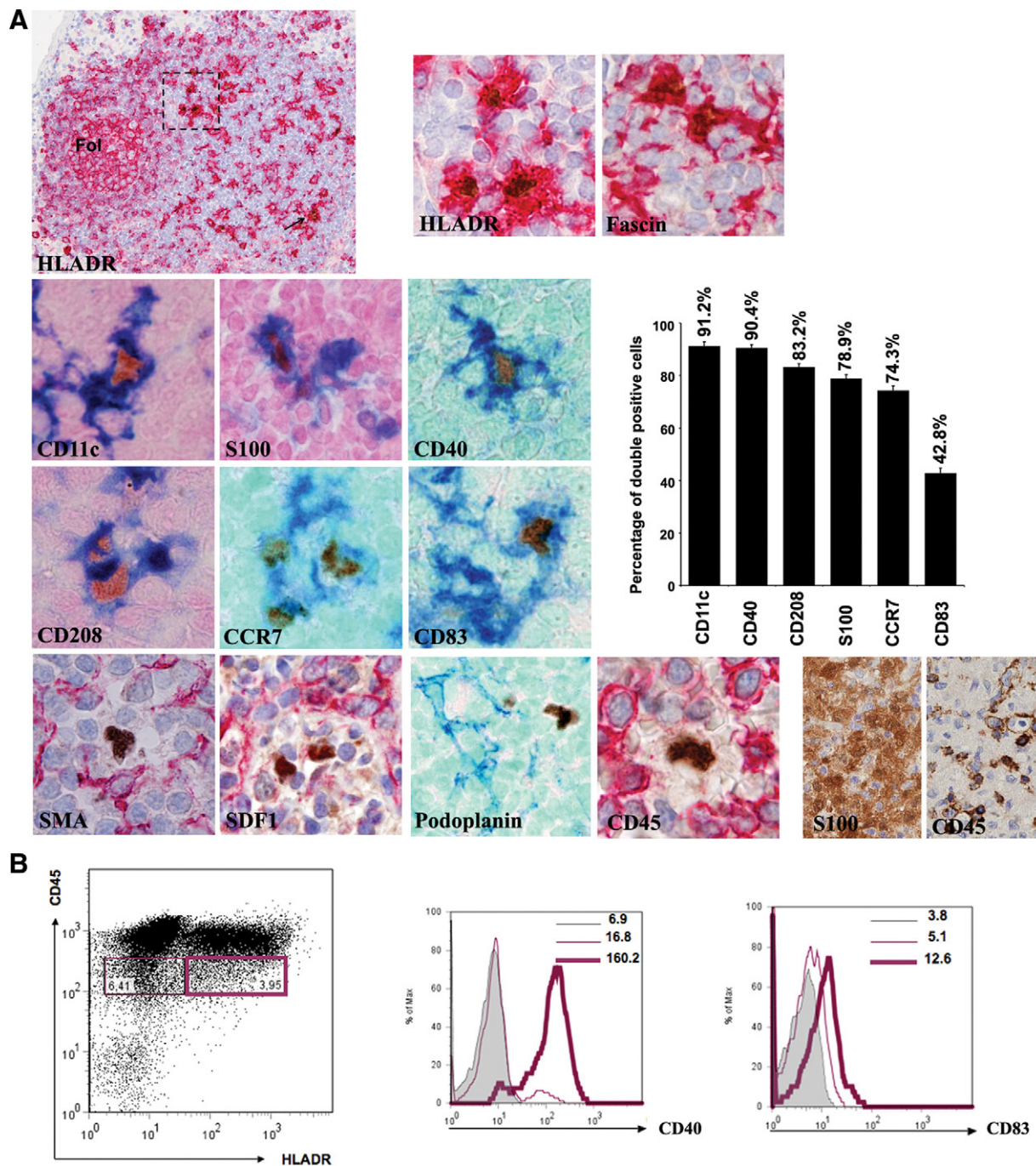
Figure 2. A: Characterization of nodal AIRE-expressing cells by double immunostains. AIRE$^+$ cells consistently express HLADR and are distributed in the outer paracortex and in the interfollicular areas (**arrow** and **dotted square**), admixed with HLADR$^+$AIRE$^-$ cells corresponding to IDCs. They coexpress other molecules normally found on IDCs, such as fascin, CD11c, and S100, as well as the DC activation and maturation antigens CD40, CD208/DC-LAMP, CCR7, and CD83. The plot shows the percentages of double-positive/total AIRE$^+$ cells. In all figures the dendritic morphology of the AIRE$^+$ cells is obvious. In the **bottom row** examples of molecules normally expressed by fibroblastic reticulum cells (smooth muscle actin [SMA], stromal cell-derived factor-1 [SDF1], and podoplanin) and lacking on the AIRE$^+$ cells are shown. AIRE$^+$ cells are also negative for CD45; this finding is not in contrast with their dendritic cell nature, because IDCs in dermatopathic lymphadenitis are composed of S100$^+$ IDCs that largely lack CD45, as shown in the **bottom right** serial sections. In double stains AIRE is developed in brown with diaminobenzidine/hydrogen peroxide, whereas the other antigen is developed either in blue or red, and nuclear counterstain with methyl green or hematoxylin, respectively. All figures, original magnification: ×40, except for the upper left figure (×4) and the lower right two figures (×20). **B:** Dot plot of cell suspension (**left**) obtained from mesenteric LNs after low-density gradient enrichment shows distribution of the CD45^{low}HLADR^{high} (purple heavy line) in comparison with CD45^{low}HLADR$^-$ (purple normal line). The CD45^{low}HLADR^{high} cell fraction displays higher level of CD40 (**center**) and a light increase of CD83 (**right**). Gray histogram represents the isotype control. Numbers indicate the mean fluorescence intensity for each population. Representative profiles of three independent experiments are shown.

was mainly supported by negativity for CD45; because of divergent data on the phenotype, these studies showed different conclusions on the origin of these cells, which were considered to be either a subset of fibroblastic reticulum cells (podoplanin/gp38$^+$, VCAM-1$^+$, ER-TR7$^+$, and UEA-I$^+$)[13] or a subset of LN stromal cells (gp38$^-$ and ER-TR7$^-$), sharing some features with medullary thymic epithelial cells (EpCAM$^+$ and PD-L1$^+$).[14]
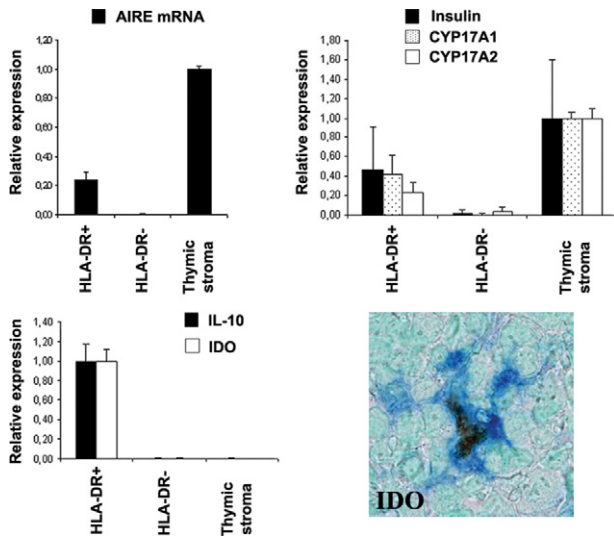
Figure 3. Nodal AIRE+ cells express tissue-restricted antigens and show a tolerogenic phenotype. Quantitative RT-PCR analysis on cell suspensions from mesenteric LNs previously enriched for AIRE expressing DCs (**top left**): the enriched HLADR+ but not the HLADR− cell fraction expresses AIRE mRNA and the AIRE-dependent tissue-restricted antigens insulin, CYP17A1, and CYP21A2 (**top right**). Quantitative RT-PCR shows that the HLADR+AIRE+-enriched cell fraction has an increased expression of IDO and IL-10 transcripts (**bottom left**), and double immunostain confirms the coexpression of IDO by the AIRE+ cells, suggesting a tolerogenic phenotype (**bottom right**). mRNA is expressed as relative gene expression levels. Averages and SEs (bars) of three independent experiments are shown. For double stain, AIRE is developed in brown with diaminobenzidine/hydrogen peroxide; IDO is developed in blue; nuclear counterstain is with methyl green. Original magnification, ×40.

Using double immunostains we have shown that the AIRE-expressing cells in human LNs are also negative for CD45 common epitopes and isoforms (RA, RB, RC, and RO) and consistently positive for HLADR; in addition, they expressed fascin, but lacked typical markers of LN stromal cells and fibroblastic reticulum cells, such as vimentin, smooth muscle actin, desmin, CXCL12/stromal cell-derived factor-1, and podoplanin/gp38.[17,18] Fascin, an actin-bundling protein that regulates cytoskeleton rearrangement and development of dendrites in various cell types, is expressed at high levels in dendritic cells (DCs) and mature interdigitating dendritic cells (IDCs).[19] Because AIRE+ nodal cells displayed an obvious dendritic morphology indistinguishable from that of IDCs, we hypothesized that they could represent a special subset of DCs. Remarkably, negativity for CD45 molecules does not contradict the DC nature of these AIRE+ cells,[20] and we showed that IDCs in dermatopathic lymphadenitis are largely negative for CD45 as well. Moreover, normal development of DCs has been reported in *CD45*^null mice and *CD45*^−/− DCs show higher levels of expression of costimulatory molecules (CD40, CD80, and CD86) and MHC-II.[21] Double immunostains for myeloid and plasmacytoid DC-associated antigens demonstrated that a high percentage of AIRE+ cells coexpressed CD11c and S100, as well as CD40, CD208/DC-LAMP, CCR7, and CD83. In keeping with these observations, high levels of CD40 and a light increase in CD83 density were detected among CD45^low^MHC-II^high LN cells after low-density gradient enrichment. Taken together, these data strongly support the notion that AIRE+ cells in human LNs

represent a subset of myeloid DCs related to IDCs, with a mature/activated phenotype. RNA transcripts encoding AIRE have been detected in DC subtypes obtained *ex vivo* and derived from culture, both in mice and humans,[11,22,23] but a functional role of the protein in DCs has been questioned.[5] Interestingly, low levels of AIRE expression have been shown in human monocyte and monocyte-derived DCs,[11] suggesting the possibility of the existence of circulating precursors of extrathymic AIRE+ DCs.[3,24] However, in keeping with data from Klamp et al[8] using mRNA analysis, in our study we failed to detect AIRE+ cells in the spleen, a secondary lymphoid organ devoid of afferent lymphatics,[16,17] thus suggesting that AIRE+ cells most likely migrate to lymph nodes along lymph vessels.

The murine LN stromal AIRE+ cells express tissue-specific antigens only partially overlapping with those expressed by thymic epithelial cells and are capable of inducing tolerance in autoreactive T cells.[13,14] To further analyze the functional properties of human AIRE+ DCs, we separated mesenteric LNs using a low-density gradient, followed by negative selection of monocytes, B-cells, and immature DCs and positive selection for HLADR. By this approach, we enriched for a cell population that express AIRE mRNA. Notably, only the AIRE-enriched HLADR+ cell fraction was found to express the AIRE-dependent antigens insulin, CYP17A1, and CYP21A2, albeit at lower levels than those observed in CD45-depleted thymic epithelial cells.

There is increasing evidence that DCs play a key role in the establishment and maintenance of immunological tolerance.[25] Thymic DCs contribute to the deletion of self-reactive T cells and to the generation of natural T-regulatory cells,[26] whereas peripheral tolerogenic DCs have been suggested to mediate deletion of self-reacting T lymphocytes and/or favor expansion of induced T-regulatory cells.[27] It has been shown previously that peripheral tolerogenic DCs express IDO and IL-10.[28,29] We hypothesized that AIRE+ DCs could represent a peculiar subset of DCs involved in the induction of peripheral tolerance. By immunohistochemical analysis, we demonstrated that the majority (92.5%) of AIRE+ DCs express IDO. Furthermore, increased expression of IDO and IL-10 transcripts was demonstrated by real-time PCR in the HLADR+CD14−CD19−CD209− nodal cell fraction.

In conclusion, we found that AIRE+ DCs are regularly located in peripheral lymphoid tissues in the area that represents the site of entry of lymph-derived antigens and dendritic cells and of blood-derived lymphocytes through high endothelial venules. This compartment has been considered to play an important role in the initiation of immune responses,[16,17] and the AIRE+ DCs might contribute to interaction with immigrating and resident cells in immune homeostasis. The demonstration that AIRE+ DCs in peripheral human lymph nodes express tissue-specific antigens and tolerogenic molecules suggests a potential role in preventing autoimmune diseases and mediating tolerance to tumor antigens.

Acknowledgments

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