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The Autoimmune Regulator (Aire) transactivates *HLA-G* gene expression in thymic epithelial cells

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Summary

The Autoimmune Regulator (Aire) protein coordinates the negative selection of developing thymocytes by inducing the expression of hundreds of tissue-specific antigens within the thymic medulla, which is also a primary site of the expression of the immune checkpoint HLA-G molecule. Considering the immunomodulatory properties of Aire and HLA-G, and considering that the role of the constitutive thymus expression of HLA-G has not been elucidated, we studied the effect of *AIRE* cDNA transfection on HLA-G expression in 4D6 thymic cells and in the HLA-G-positive JEG-3 choriocarcinoma cells. Aire promoted the transactivation of *HLA-G* gene by increasing the overall transcription, inducing the transcription of at least G1 and G2/G4 isoforms, and incrementing the occurrence and distribution of intracellular HLA-G protein solely in 4D6 thymic cells. Luciferase-based assays and chromatin immunoprecipitation experiments performed in 4D6 cells revealed that Aire targeted at least two regions within the 5′-untranslated regulatory region (5′-URR) extending 1·4 kb from the first ATG initiation codon. The interaction occurs independently of three putative Aire-binding sites. These results indicate that the Aire-induced upregulation of HLA-G in thymic cells is likely to act through the interaction of Aire with specific *HLA-G* 5′-URR DNA-binding factors. Such a multimeric transcriptional complex might operate in the thymus during the process of promiscuous gene expression.

Keywords: Aire; central tolerance; expression regulation; HLA-G; thymus.

Introduction

The thymic education represents an essential step to maintain T-cell homeostasis and prevent autoimmunity,[1,2] as the thymus provide a suitable microenvironment for the occurrence of positive and negative selection of developing thymocytes.[1,2] The major regulator of negative selection is the *AIRE* gene (Autoimmune Regulator) that encodes a transcription factor expressed particularly, but not exclusively, on the CD80^{hi} MHC-II^{hi} medullary thymic epithelial cells (mTECs) and intrathymic dendritic cells (DCs).[3,4] Aire coordinates the medullary expression of hundreds of tissue-related antigens (TRAs) from peripheral tissues, by the phenomenon known as promiscuous gene expression (PGE),[3–5] ensuring the exposure of the majority of antigens from periphery within the medullary compartment during thymic selection.[3–5] Aire is located within the nuclear speckles associated with a multimeric transcriptional complex composed by proteins related to nuclear transport, chromatin modification and transcription initiation, such as DNA-dependent protein kinase (DNA-PK); topoisomerase 2a (TOP2a) and topoisomerase 1 (TOP1) that co-localizes with super-enhancers to promote the association with Aire-containing

Abbreviations: Aire, autoimmune regulator; HLA, human leucocyte antigen; mTEC , medullary thymic epithelial cells; PGE, promiscuous gene expression

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complexes;[6] RNA polymerase II (RNAPII); Ku80 and Ku70.[7] Additionally, Aire co-localizes with CREB-binding protein, positive transcription elongation factor (P-TEFb) and ribonucleoproteins.[7,8]

Aire has several functional domains shared by nuclear proteins and transcription factors. Briefly, the N-terminal portion comprises the nuclear localization signal, an oligomerization domain homogeneously staining region, a DNA-binding domain composed of the SAND (Sp100, Aire-1, NucP41/75, DEAF-1) proteins, and two plant homeodomains zinc finger-type (PHD1 and PHD2), which are related to protein–protein interactions and bind to nucleosomes and histones H3, discriminating methylated (H3K4me3,2,1) and non-methylated histones.[9,10] Moreover, the PHD1 domain is associated with the binding to DNA-PK, promoting changes in chromatin structure and DNA double-strand breaks.[11,12] The Aire C-terminal portion has a transcription activator domain involved in the binding to P-TEFb and TRA genes, phosphorylating the Serine 2 in RNAPII, and leading to productive elongation and transcription of mRNAs.[8,12,13]

Beyond PGE, Aire has been associated with several other important cellular functions, such as the modulation of differentiation programmes of mTECs,[14] regulation of expression of several intrathymic chemokines acting on the recruitment of thymocytes, mTECs and DCs to sites of occurrence of the negative selection;[15,16] induction of apoptosis;[17–19] and induction of subpopulations of natural regulatory T-cells.[20,21] The mechanisms regulated by Aire during the central tolerance are essential to ensure the immunological homeostasis; however, they are not enough to prevent the release of autoreactive clones of T-lymphocytes in periphery. Thus, the thymus expression of regulatory molecules represents an essential mechanism for the control of the immune responses in tissues.[22]

The non-classical class I human leucocyte antigen-G (HLA-G) was initially identified in the placenta,[23,24] being related to the acceptance of the fetus by the maternal organism.[24,25] HLA-G is also constitutively expressed on chorionic and corneal tissues,[26–28] erythroid and endothelial precursors,[29] fetal liver and bone marrow mesenchymal stem cells,[30] and thymus cells.[31,32] In contrast to classical HLA class I molecules (HLA-A, -B and -C) that are expressed on all nucleated cells, HLA-G is not involved in antigen presentation and has a limited protein polymorphism (the IMGT database 3·33·0 lists 61 alleles, coding only 19 proteins). These alleles give rise to several isoforms through alternative splicing of the primary mRNA transcript, yielding at least four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three soluble (HLA-G5, G6 and -G7) proteins.[33–36] The expression of HLA-G is also inducible in allografts without rejection and under many pathological conditions, such as cancer, viral infections, autoimmune and inflammatory diseases.[25,33]

HLA-G is considered to be an immune checkpoint molecule, interacting with at least three inhibitory receptors: (i) ILT-2 (CD85j/LILRB1) is expressed on subsets of T-lymphocytes and NK cells, and on monocytes/macrophages, B-lymphocytes and DCs;[37] (ii) ILT-4 (CD85d/LILRB2) is expressed on monocytes/macrophages and DCs;[37] and (iii) p49/KIR2DL4 is expressed on NK cells and CD8+ T-lymphocytes. KIR2DL4 is a specific receptor for HLA-G; however, its precise function is still controversial.[38] HLA-G modulates the immune responses by inhibiting: (i) the cytotoxic activities of NK cells and cytotoxic CD8+ T-lymphocytes;[25,39,40] (ii) CD4+ T-cell proliferation;[41] and (iii) proliferation, differentiation and secretion of immunoglobulins of activated B-cells.[42] Otherwise, HLA-G inhibits the trans-endothelial vascular adhesion process,[43] promotes polarization of the immune response to a Th2 profile,[44] induces tolerogenic antigen-presenting cells,[45] regulatory T-cells through trogocytosis,[46] and differentiation of naïve $CD4^+$ or $CD8^+$ T-cells into regulatory T-cells.[47]

The regulation of HLA-G expression is unique and tissue-specific. HLA-G may be induced under stress conditions[48,49] and by a number of factors in the microenvironment, such as IL-10,[50] IFN,[51] glucocorticoids[52] and progesterone.[53,54] The *HLA-G* gene transcription regulation differs from other HLA class I genes.[55] The promoter region extending 200 bp prior to the first ATG initiation codon (nucleotide + 1) presents distinct features from those observed for classical HLA class I genes.[56,57] The *HLA-G* proximal promoter region has no regulatory elements that respond to IFN-γ and NF-κB, and the W/S-X-Y motif, which assembles the MHC-enhanceosome, is disrupted. Some alternative regulatory response elements have been described within the 1·4-kb upstream segment from the first ATG, including functional binding sites for CREB-1 (−1387 bp to −1371 bp/-941 bp to −935 bp/−777 bp to −771 bp), IRF-1 (−745 bp to −754 bp), HSF-1 (−464 bp to −453 bp) and progesterone receptor (−52 bp to −58 bp), which all activate *HLA-G* transcription. More recently, a cis-regulatory element 12 kb upstream of *HLA-G* with enhancer activity (Enhancer L) was demonstrated to specifically control HLA-G expression in trophoblasts.[58] At the opposite, functional response elements for RREB-1 (−1378 bp to −1358 bp/−157 bp to −143 bp/−59 bp to −54 bp)[59] and Gli-3 (−1116 bp to −1108 bp),[60] and a LINE1 family member located −4 kb from ATG[61] were shown to downregulate *HLA-G* expression. Additionally, the expression of the *HLA-G* gene is regulated at the epigenetic level through histone modifications and methylation of CpG motifs within the proximal 5′-regulatory region.[62,63] Otherwise, HLA-G is controlled at the post-transcriptional level by mechanisms acting on the 3′-untranslated region *(UTR)*, more particularly through the binding of microRNAs.[64] Interestingly, the 5′-upstream regulatory region (*5′-URR*) and the *3′-UTR* exhibit a

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significant polymorphism, which may affect the binding of regulation factors and, thus, influencing HLA-G expression.[65,66]

Considering that: (i) HLA-G1 is expressed on the surface of mTECs, in thymic stromal cells of the cortical-medullary junction and in the subcapsular region;[31,67] (ii) the soluble HLA-G5 isoform is found within the thymus medullary compartment;[32] (iii) the expression of HLA-G occurs in thymic compartments and on cell subpopulations that are also positive for Aire;[32] (iv) there is a positive correlation between the transcriptional profiles of *AIRE* and the murine functional homologue of *HLA-G* (*Qa-2/H2-Q7*) during the fetal period of thymus ontogeny,[68] we investigated whether or not the regulation of HLA-G may be under the influence of Aire, which could be a potential transcription factor for the transactivation of *HLA-G* gene.

Materials and methods

Plasmids

The pcDNA3·1 plasmid encoding for Aire (1·6 kb) (NM_000383·3) (pcDNA3·1-*AIRE*), containing a sequence encoding a hexahistidine-tag, was kindly provided by Dr Matthieu Giraud (Institut Cochin, Paris) and Dr Diane Mathis (Harvard University, USA).[7] The *PROMO-G010101a* and *PROMO-G010102a*, which are the most frequently observed haplotypes worldwide of the *HLA-G* upstream regulatory region (1438 bp segment from the first translated ATG), were isolated by polymerasechain reaction (PCR; forward *5′-AAGCTTCACAAGAATGAG GTGGAGC* and reverse *5′-CGCGGATCCTTGGCGTCTGG* primers) performed on HLA-G-typed genomic DNAs and cloned first in pUCm-T vector (Bio Basic, Markham, ON, Canada) and then in PGL3-basic vector (KpnI/BamHI). The mutated haplotype *PROMO-G010101aMut* was generated by double-strand DNA synthesis (GeneCust Europe, Dudelange, Luxembourg) introducing mutations at three putative Aire-binding sites: the consensus sequence *CTGGTT* at the binding sites BS1 (*CTGGTTCTAA*), BS2 (*CTGGTTTCTC*), BS3 (*CTGGTTGCAA*) was substituted for *AAAAA* sequence, as follows: BS1 (*AAAAACTAA*), BS2 (*AAAAAATCTC*) and BS3 (*AAAAAAGCAA*). The mutated haplotypes were inserted within the Hind III restriction site of PGL3-basic vector (Promega, Madison, WI).

Human cell lines

The choriocarcinoma cell line JEG-3 (American Type Culture Collection) was grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 250 mg/l fungizone (Invitrogen, Carlsbad, CA) and 10 mg/l gentamicin (Invitrogen). The mTEC line 4D6 (provided by Dr Matthieu Giraud)[7] was maintained in RPMI 1640 (Sigma-Aldrich, Saint-Louis, MO) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 10% heat-inactivated fetal calf serum, 250 mg/l fungizone (Invitrogen) and 10 mg/l gentamicin (Invitrogen, Carlsbad, CA). All cultures were maintained at 37° and 5% CO_2. Cells were routinely tested for absence of contamination with mycoplasma (plasmoTest, InvivoGen, San Diego CA).

Transient transfections

The day before the transfection, 4×10^5 cells were seeded in six-well plates. When at 50%–60% confluence, each cell culture well was transfected using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions, with a DNA mixture including a total of 2·5 μg of plasmids. After 48 hr of transfection, cells were harvested and prepared for further experiments.

Western blot

The Aire expression following transfection of pcDNA3·1-*AIRE* (or empty pcDNA3·1-control vector) in 4D6 and JEG-3 human cell lines was validated by Western blotting (see Supplementary material, Fig. S1). Transfected cells were collected and lysed in lysis buffer (1% NP-40, 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 2 mmol/l EDTA and 10% glycerol). After centrifugation at 16 000 *g* at 4° for 30 min, supernatants were transferred to Eppendorf tubes (Dutscher, Brumath, France). Equal amounts of cell lysates (50 μg) were normalized by the micro-BCA assay (Pierce, Rockford, IL) and separated in 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. All samples were electroblotted onto Hybond-C extra membrane (Amersham, Little Chalfont, UK) and blocked by incubation with Tris-buffered saline (TBS) and 5% non-fat dry milk for 30 min. After blocking, membranes were washed three times in TBS solution containing 0·2% Tween-20, then probed with the primary antibodies: anti-Aire-1 (sc-373703) (Santa Cruz Biotechnology, Santa Cruz, CA) (mouse mAb specific for amino acids 246–545 in the C-terminal portion of Human Aire-1 protein) at the 1 : 500 dilution and anti-α-tubulin (Sigma-Aldrich, Saint-Louis, MI) (mouse mAb IgG1) at the 1 : 2000 dilution overnight at 4° (internal control), then washed three times in TBS containing 0·2% Tween-20. The membranes were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-mouse IgG directed against heavy and light chains (H&L) and washed three times in TBS containing 0·2% Tween-20. The membranes were treated with West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 1 min and exposed to Kodak Biomax MR film (Sigma-Aldrich). The total hepatocyte cell lysate (Hep G2 Cell Lysate - sc-2227) (Santa Cruz) was used as a positive control for Aire.

RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was isolated using TRizol® reagent (Invitrogen), according to the manufacturer's instructions, and treated with DNAse (deoxyribonuclease I amplification grade, Invitrogen). Reverse transcription was conducted on 1 μg RNA using the High-Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions.

Duplex quantitative real-time PCR was performed in triplicate, as previously described;[61] with ABI Prism 7000 SDS (Applied Biosystems) using TaqMan Universal PCR Mix, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; TaqMan Endogenous Assay) as an endogenous control, *HLA-G* specific primers (forward 5′-*CTGGTTGTCCTT GCAGCTGTAG* and reverse 5′-*CCTTTTCAATCTGAGC TCTTCTTTCT*) and a probe located in exon 5 that targets all known HLA-G mRNAs (*HLAEX5*: 5′-*CACTG-GAGCTGCGGTCGCTGT*). For HLA-A and HLA-E genes, the amplification was performed using the Taqman inventoried sets of specific primer/probes (HLA-A: ID: Hs01058806_g1; forward 5′-*GCTACTCTCAGGCTGCAAG-CAGTGA* - FAM/MGB; and HLA-E: ID: Hs03045171_m1; forward 5′-*GCTTCACCTGGAGCCCCCAAAGACA* - FAM/MGB). The PCR conditions for all genes were: 50° for 2 min, 95° for 10 min and 40 repetitions at 95° for 15 seconds followed by 60° for 1 min.

HLA-G mRNA quantification was performed relative to amounts of *HLA-G* transcripts in *HLA-G*-positive JEG-3 using the comparative Ct method:[61] $Ct = Ct_{HLA\text{-}G} - Ct_{GAPDH}$: $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{JEG\text{-}3}$; relative HLA-G expression = $2^{-\Delta\Delta Ct}$. Similarly, for the assessment of relative gene expression of HLA-A, HLA-E and AIRE, the comparative Ct method was used: $\Delta Ct = Ct_{target} - Ct_{GAPDH}$: $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{JEG\text{-}3}$; relative expression = $2^{-\Delta\Delta Ct}$. At least three independent experiments were performed.

Semi-quantitative RT-PCR to identify alternatively spliced *HLA-G* transcripts was carried out with a Mastercycler® pro vapo.protect™ (Eppendorf, Montesson, France) in a total volume of 50 μl containing 2 μl of cDNA, 200 μM each dNTP (Amersham Pharmacia-Biotech), 25 mM of each primer (ESGS-Groupe Cybergene, Saint-Malo, France), 10 μl of 10 × PCR buffer and 2·5 U of Taq polymerase (Perkin Elmer). *HLA-G*-specific amplification used the primer pair G.257-Forward (exon 2; 5′-*GGAAG AGGAGACACGGAACA*) and G.1004-Reverse (exon 5 and exon 6 junction; 5′-*CCTTTTCAATCTGAGCTCTTCTTT*) during 35 cycles. PCR cycle conditions were 2 min at 94°C, 1 min 30 at 58°, 2 min at 72°, followed by 1 min at 72°. *β-ACTIN* cDNA amplification was performed using primer set Actin-Forward (5′-*ATCTGGCACCACACCTTCTACA ATGAGCTGCG*) and Actin-Reverse (5′-*CGTCATACTCC TGCTTGCTGATCCACATCTGC*) during the last 16 cycles of PCR and was used as internal standard. The expected major amplicons were as follows: 0·75 kb for HLA-G1, 0·45 kb for HLA-G2/G4, 0·17 kb for HLA-G3 and 0·84 kb for *β-ACTIN*.

Reverse transcriptase-PCR analyses of *AIRE* and *KERATIN 14* transcriptional activities were carried out with Aire-Forward (5′-*CAGAGAGCTGTGGCCATGT*), Aire-Reverse (5′-*CCTGGATGCACTTCTTGGA*), Keratin-Forward (5′-*CCAGCTCAGCATGAAAGCATC*), Keratin-Reverse (5′-*CCATCGTGCACATCCATGAC*) primers during 40 cycles (2 min at 94°, 1 min at 54°, 2 min at 72°) followed by 1 min at 72°.

The discrimination of PCR products was conducted in 1.5% agarose gel electrophoresis.

Flow cytometry

JEG-3 and 4D6 cells (2×10^5), transfected or not with *AIRE*, were harvested and washed in a solution containing 1 × phosphate-buffered saline (PBS), 0·1% bovine serum albumin. HLA-G cell surface expression was evaluated with MEM-G/9-PE-conjugated mAb (IgG1 mouse anti-human) (Exbio, Praha, Czech Republic), which recognizes the HLA-G1 isoform associated with β2-microglobulin, or G233 (Exbio).[69] Isotype control IgG1-PE-conjugated was used at the same concentration as the primary antibody. Incubation was performed at 4° for 30 min, followed by two washes and fixation with 1 × PBS and 0·5% formaldehyde 37%. Cells were analysed by a Flow Epics XL cytometer (Beckman Coulter, Brea, CA), using the Expo32 ADC 1·1C software (Beckman Coulter) for data acquisition. Specific fluorescence indexes (SFIs) were calculated by dividing the mean fluorescence of specific antibody by mean fluorescence obtained with isotype control antibody. A SFI of 1·5 was considered to be positive.

Immunocytochemistry

Thymus 4D6 and JEG-3 cell suspensions, transfected or not with *AIRE*, were placed on glass slides (Superfrost®PLUS, Thermo Scientific) by cytospin using a cytocentrifuge (Shandon CytoSpin 3, Thermo Scientific). 4D6 cell suspensions containing $7{\cdot}5 \times 10^4$ cells/100 μl were subjected to 10·16 G rotation for 1 min, while $2{\cdot}5 \times 10^4$ cells/100 μl JEG-3 cell preparations were rotated at 18·06 G for 1 min, then immersed in ice-cold acetone for 15 min at −20°. The slides were dried at room temperature and then stored at −20° until use. The immunocytochemistry reactions were performed using anti-HLA-G mAbs 4H84 (IgG1 mouse anti-human; Exbio), which recognizes the α1 domain common to all HLA-G isoforms, or MEM-G/2 (IgG1 mouse anti-human; Exbio). For the distinction of non-specific labelling, IgG1 isotype control was used under the same conditions. All antibodies were diluted 50 ×. As positive control for HLA-G, cytotrophoblast slices from human placentas of the third

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trimester of gestation were used. Immunostaining for HLA-G molecule was detected using the Ultratech HRP Streptavidin-Biotin Universal Detection System kit (Immunotech-Coulter, Beckman Coulter) according to the manufacturer's instructions. Antibody fixation was revealed by the AEC+ Substrate Chromogen kit (Dako). The images were acquired under a Nikon Microscope Eclipse 80i microscope coupled to a digital camera (Nikon, Melville, NY) and analysed by Image J software version 1·47 (https://imagej.nih.gov/ij/download.html). The qualitative analysis consisted in the determination of the occurrence of HLA-G, its cell location and intensity pattern staining. The quantitative analysis consisted in calculating the expression intensity (staining area) of each sample, by processing and transforming the image into binary scale (eight bits), establishing the common threshold and calculating the staining area in pixels. The results were expressed in Arbitrary Units (AU), representative of the ratio between the intensity of the specific staining area and the background in pixels/μm divided by the number of counted cells (200 cells/treatment).

ELISA assays

The sHLA-G in culture supernatants (80 μl) was quantified by sandwich ELISA using mAb anti-HLA-G MEM-G/9 (Exbio) and anti-β2-microglobulin (DAKO, Glostrup, Denmark) as capture and detection antibodies, respectively, as previously described.[70]

Dual-Luciferase Reporter activity assays

For the Dual-Luciferase® Reporter (DLR™) Assay System (Promega), JEG-3 and 4D6 cell lines were co-transfected with a DNA mixture including a total of 2·5 μg of DNA plasmids: (I) assay efficiency and internal control: pGL3-*promoter* vector containing an SV40 promoter upstream to *Luciferase* gene (pGL-3-*PROMO*); (II) controls for assay background: empty pGL3-*basic* and empty pcDNA3·1 vectors; (III) HLA-G 5′-URR haplotypes: pGL3-*G010101a* and pGL3-*G010102a* corresponding to pGL3-basic constructions with the HLA-G 5′-URR upstream to the firefly Luciferase gene; and (VI) *AIRE*-containing plasmid pcDNA3·1-*AIRE* and empty pcDNA3·1. Cell lines were co-transfected with appropriate mixture of plasmids and 50 ng of *Renilla Luciferase* pRL-CMV (control for transfection efficiency) in a final volume of 0·5 ml. This system provided an estimation of the activity of the 5′-URR of *HLA-G* (which is cloned upstream to the firefly *Luciferase* gene) according to the presence or absence of *AIRE* in co-transfected cells. The activities of firefly (*Photinus pyralis*) and renilla (*Renilla reniformi*) luciferase were measured sequentially from a single sample using a luminometer (BMG FLUOstar OPTIMA Microplate Reader, BMG Labtech). Assays were run in triplicate with at least three independent experiments. To finally determine the effect of Aire on the HLA-G 5′-URR activity, firefly luciferase values were first normalized to those of Renilla luciferase values, and then to the normalized luciferase expression of the empty pGL3-*PROMO* vector.

Immunoprecipitation of chromatin assays (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT High-Sensitivity® Kit (Active Motif, La Lupe, Belgium) according to the manufacturer's instructions, with anti-AIRE-1 (sc-373703; Santa Cruz, CA, USA), anti-6× His-tag (Abcam, ab125262) and isotype control. Briefly, 4D6 cell line was transfected by electroporation (Gene pulser II system, Bio-Rad) and then exposed to 1% formaldehyde for cross-linking proteins and DNA together, followed by chromatin sonication into fragments of 300–600 bp size. Cross-linked chromatin was immunoprecipitated with the indicated antibody. The antibody–protein–DNA complexes were purified using Protein-G-coupled beads in purification columns. The final DNA from ChIP and input chromatin (PCR control) were then used as templates in PCR using primers set: (I) G-836 Reverse (*5′-GCCCTAAGTTTCCGTGTGAGTCCA*) and G-1119 Forward (*5′-CGCTGGGTGTTCTTTGCAGTTG*) (BS1 segment); (II) G-166 Reverse (*5′-CCCGACACAGGTTAGGAGAA*) and G-364 Forward (*5′-GACCTTGCCGAGGG TTTCT*) (BS2 segment); (III) G-29 Reverse (*5′-GTCTG GGGAGAATGAGTCCGG*) and G-187 Forward (*5′-CCCG CGTTGGGGATTCTCTC*) (BS3 segment).

In silico identification of Aire putative binding sites

In silico analysis for the identification of putative binding sites for Aire in the *HLA-G* gene (gene sequence NG_029039), was performed using the PROMO platform version 3·0·1 available online (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_6·4).[71]

Statistical analysis

All statistical tests were performed using the GraphPad Prism software version 5·0 (GraphPad Software, San Diego, CA). The non-parametric two-tailed Mann–Whitney and Wilcoxon tests were used. Significance was defined as $P < 0{\cdot}05$ at a confidence interval of 95%.

Results

Aire boosts *HLA-G* gene expression in epithelial thymic cells

To evaluate the ability of Aire to participate in the transcriptional activation of *HLA-G* gene, 4D6 and JEG-3 cell lines were transiently transfected with pcDNA3·1-*AIRE* or empty

pcDNA3. Aire expression was validated by Western blot analysis (Fig. S1). Using real-time RT-PCR, we observed a significant increase in the total *HLA-G* transcript levels in *AIRE*-transfected 4D6 thymic cells compared with the control vector pcDNA3·1 ($P = 0{\cdot}015$; Fig. 1a). The concomitant expression of an Aire-dependent gene (*KERATIN 14*) in 4D6 cells confirmed the functionality of the transfected *AIRE* (Fig. S2). On the other hand, no differences in the *HLA-G* transcript levels between the transfected groups were observed with JEG-3 cells (Fig. 1b).

Regarding the alternative splicing of *HLA-G* primary mRNA, qualitative changes were observed in three out of five independent transfections of 4D6 cells with AIRE, showing the induction of at least *G1* and *G2/G4* transcripts (0·75 kb and 0·45 kb bands, respectively), in addition to the constitutively expressed *HLA-G3* transcript (0·17 kb band; Fig. 2a). In JEG-3 cells, the pattern of alternative splicing was unchanged (Fig. 2b). Otherwise, the transcriptional activation of *HLA-G* in the presence of Aire in thymic cells seemed to be HLA-G-specific, as no changes in the transcriptional levels of HLA-E and HLA-A were observed (Fig. 3).

Aire enhances intracellular HLA-G protein expression

To examine the impact of Aire on the HLA-G protein expression, immunocytochemistry analysis with 4D6 cells transfected with *AIRE* showed an increased staining area positive for HLA-G molecules (4H84 antibody) compared with the non-transfected 4D6 cells or transfected with control pcDNA vector (Fig. 4a). Additionally, 4D6 cells transfected with *AIRE* showed a slightly different stain pattern with increased staining intensity and cell localization scattering along the cytoplasm, compared with non-transfected cells or transfected with control pcDNA vector, in which the HLA-G-positive cell localization was restricted to a small perinuclear area forming beam-like structures. HLA-G detection was confirmed using a second anti-HLA-G antibody (MEM-G/2; data not shown). The expression of HLA-G in JEG-3 cells was similar for both groups, transfected or not, characterized as previously reported,[62] by the presence of heterogeneous JEG-3 cell population, which is positive and negative for HLA-G molecule (Fig. 4b).

Regarding the cell surface expression of HLA-G, flow cytometry performed with MEM-G/9 antibody revealed

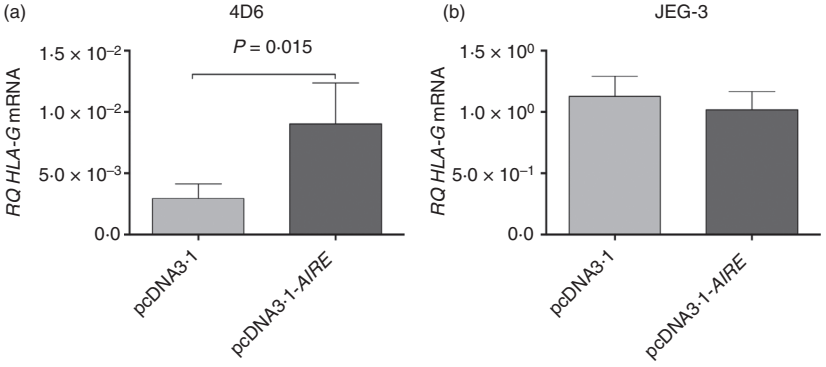

Figure 1. HLA-G gene expression in (a) 4D6 human thymic cell line and (b) JEG-3 human choriocarcinoma cell line transfected with *AIRE* or not. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) targeting all *HLA-G* mRNAs forms (at least five independent experiments); RQ = Relative Quantification to the mRNA expression in JEG-3 cells (assigned a value of 1). Significance level adopted ($P < 0{\cdot}05$) is represented with (*). Non-parametric Mann–Whitney test was used.

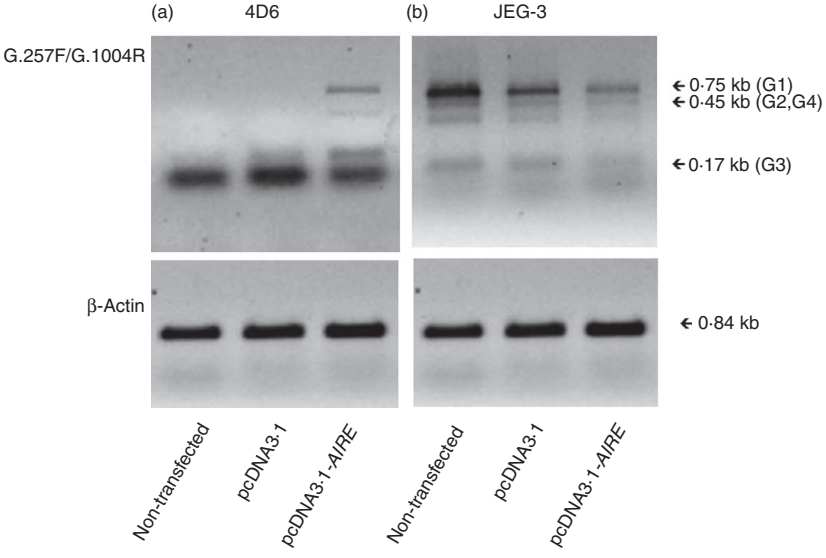

Figure 2. Alternative splicing of HLA-G transcripts in (a) 4D6 human thymic cell line and (b) JEG-3 choriocarcinoma human cell line transfected or not with *AIRE*. The BET-coloured gels (1·5% agarose) are representative of at least three independent experiments for 4D6 and five for JEG-3. Amplifications were performed on cDNA using *HLA-G* primer set G.257F (exon 2)/G.1004R (exon 5-exon 6). *β*-actin indicates reverse transcriptase-polymerase chain reaction (RT-PCR) products used as internal controls.

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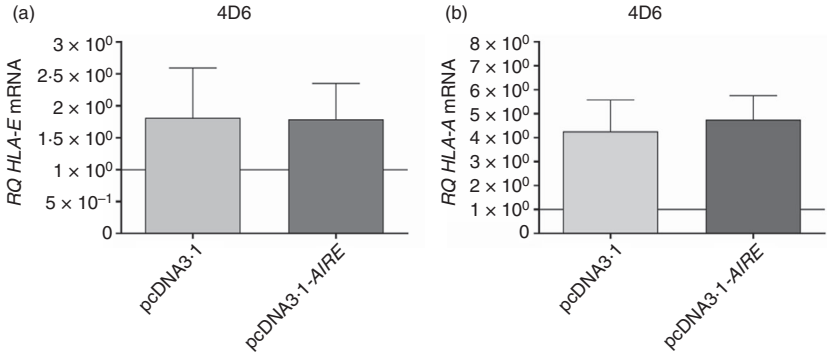


Figure 3. (a) HLA-E and (b) HLA-A gene expression in 4D6 human thymic cell line transfected with *AIRE* or not. RQ = Relative Quantification to the mRNA expression detected by real-time quantitative polymerase chain reaction (PCR) in 4D6 cells (at least five independent experiments). Significance level adopted ($P < 0{\cdot}05$) is represented with (*). Non-parametric Mann–Whitney test was used.

no differences in the presence or absence of Aire, both in 4D6 (Fig. 5a) and JEG-3 cells (data not shown). The absence of HLA-G expression at the cell surface of 4D6 cells transfected or not with *AIRE* was validated using a second anti-HLA-G antibody (G233; data not shown). In addition, we observed by ELISA the absence of HLA-G secretion (shed HLA-G1 and HLA-G5) in culture supernatants (Fig. 5b).

Therefore, the Aire-mediated upregulation of *HLA-G* gene transcription in the thymic medullary epithelial cells has an impact only on the increasing and accumulation of intracellular expression of HLA-G.

Aire acts on the 5′-URR of *HLA-G*

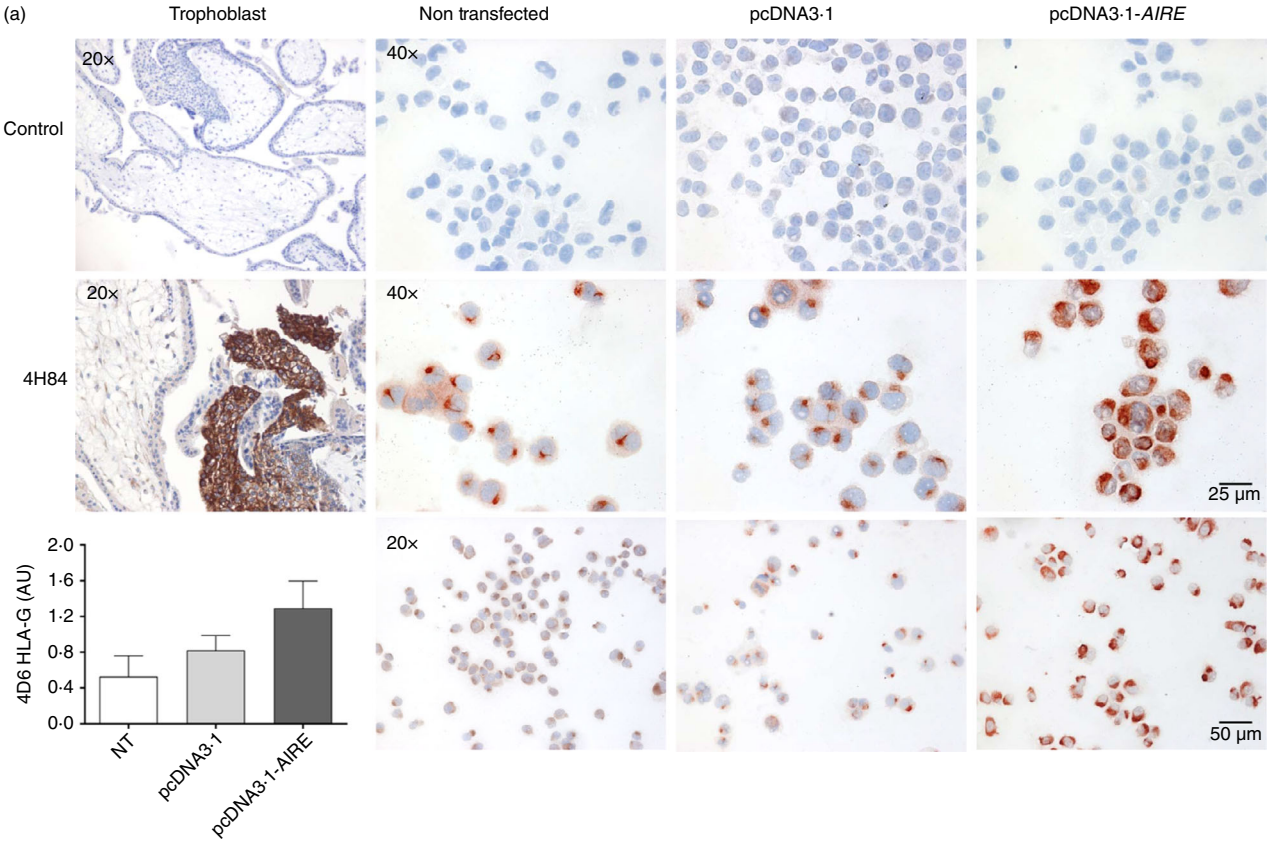
Aiming to investigate: (i) the ability of Aire in the transactivation of the *HLA-G* gene by its 5′-URR (1·4 kb upstream the ATG); and (ii) the influence of 5′-URR polymorphism in the Aire accessibility; we analysed by reporter Luciferase Reporter Activity Assay the influence of Aire in the promoter activation of two of the most frequent *HLA-G* 5′-URR haplotypes, *PROMO-G010101a* and *PROMO-G010102a*, that differ at 13 positions. Co-transfection with *AIRE* in 4D6 and JEG-3 cells provided a significant increase in luciferase activity for both haplotypes in 4D6 samples co-transfected with *AIRE* compared with the control pcDNA vector ($P = 0{\cdot}007$; Fig. 6a), while no significant change was observed in JEG-3 cells (Fig. 6b), thus confirming the cell-specific action of Aire. Interestingly, we observed an increased luciferase activity of the haplotype *G010101* compared with *G010102a* ($P = 0{\cdot}007$; Fig. 6b), but the action of Aire was independent of the haplotype.

To investigate whether Aire directly targeted *HLA-G* 5′-URR, we first searched for potential Aire-binding sites within this region. *In silico* analysis performed with the PROMO platform (*Transfac V6·4*) revealed three putative binding sites sharing the core consensus sequence *CTGGTT*. They are located outside the 5′-URR variables sites at positions -889/-880 (*CTGGTTCTAA*: binding site 1 – BS1); -343/-334 (*CTGGTTTCTC*: binding site 2 – BS2); and -55/-56 (*CTGGTTGCAA*: binding site 3 – BS3) from the first translated ATG (position 1; Fig. 7). To evaluate whether these binding sites were functional targets of Aire, we performed luciferase reporter experiments in 4D6 thymic cells aiming to compare the effect of Aire on *PROMO-G010101aMut,* where putative binding sites have been disrupted by replacing the core sequence *CTGGTT* with *AAAAA*, with the effect of Aire on the wild-type *PROMO-G010101a*. No significant differences were observed in the luciferase activity between the PROMO-*G010101a* haplotype and the PROMO-*G010101aMut* (Fig. 8), suggesting a possible indirect action of Aire on HLA-G transactivation and/or the Aire binding outside the predicted sites.

To further evaluate the association of Aire with *HLA-G* 5′-URR, the chromatin of 4D6 cell lines transfected or not with *AIRE* was immunoprecipitated and the purified DNA further analysed by PCR targeting three segments located in the distal (BS1), intermediate (BS2) and proximal (BS3) parts of the 5′-URR. ChIP assays carried out with anti-Aire and anti-Tag 6x histidine antibodies were clearly positive for at least the BS1 and BS3 regions in 4D6 cells transfected with *AIRE,* compared with those transfected with pcDNA control vector (Fig. 9a,c). Therefore, Aire binds *in situ* the HLA-G 5′-URR, but does not interact directly with the DNA within this region.

Discussion

The *AIRE* gene encodes a transcription factor involved on a multiprotein complex, coordinating the expression of hundreds of TRAs within the thymic medulla by the process of PGE, ensuring the central tolerance.[2–4] Aire controls the promoter regions of diverse TRA genes by unlike mechanisms in their corresponding tissues, raising the question of how Aire regulates a large repertoire of unlikely genes. In the present study, we demonstrated for the first time that Aire transactivates the immune checkpoint *HLA-G* gene and increases intracellular HLA-G protein expression in thymic epithelial cells. The induction of *HLA-G*, but not of *HLA-A* and *HLA-E,* and the immunoprecipitation of Aire at the *HLA-G* promoter



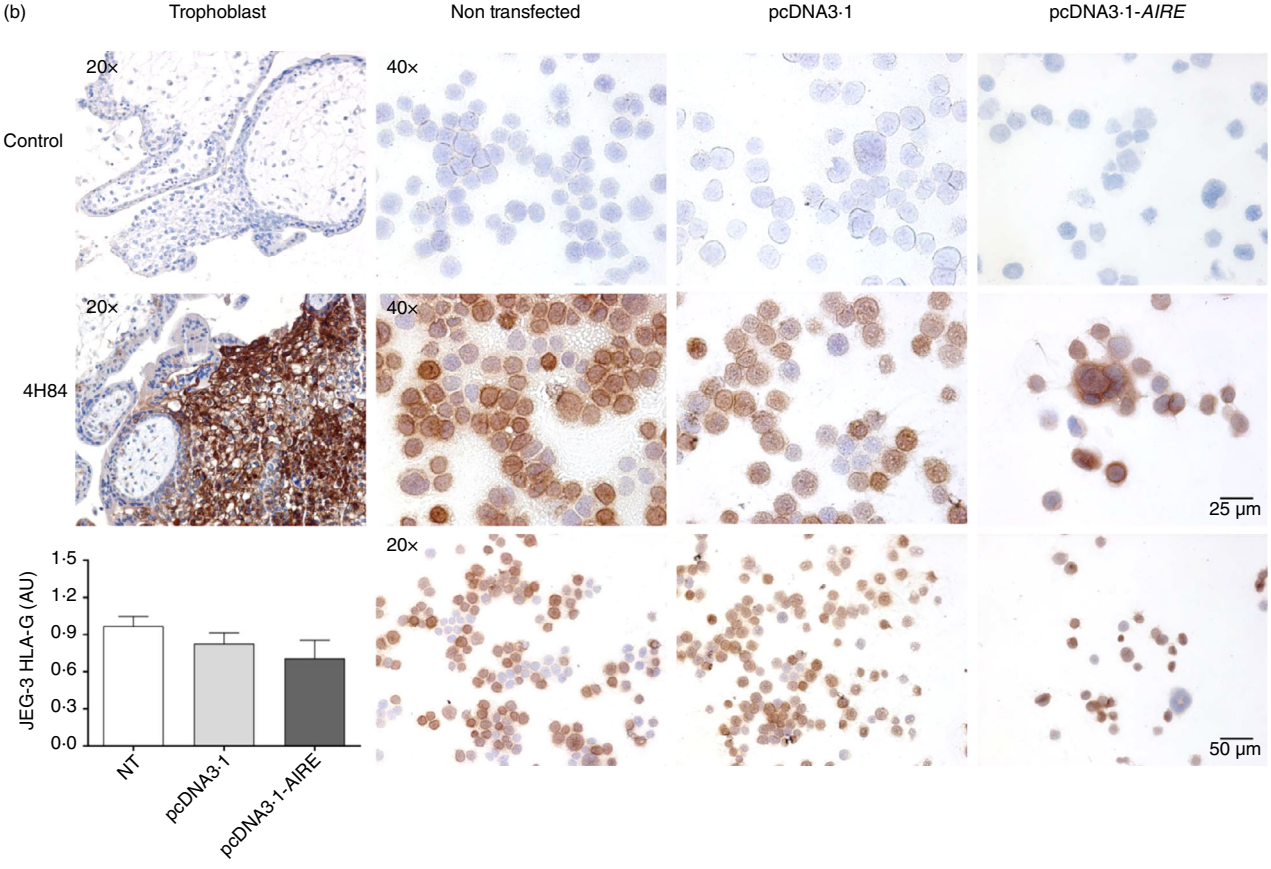


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Figure 4. HLA-G immunostaining using anti-HLA-G antibody (4H84) in (a) 4D6 human thymic cell line transfected with *AIRE* or not, and (b) JEG-3 choriocarcinoma human cell line. Images were captured using a 20 × and 40 × objective. Human cytotrophoblast sample served as a positive control (brownish staining). 'Control' indicates that anti-IgG1 antibody was used instead of 4H84. The graph indicates the mean intensity of the staining area for each condition and standard deviation. AU, arbitrary units: the ratio between the intensity of the specific staining area and the background in pixel/μm divided by the number of cells (200 cells/treatment) analysed by ImageJ software.

region suggests a synergy between HLA-G and Aire on the control of central tolerance.

We have previously observed that HLA-G expression in primary thymic culture dramatically decreases, according to the cell subculturing.[67] In addition, HLA-G expression decreases during cultivation of renal cell carcinoma cells,[72] and a switch of HLA-G1 to HLA-G2 transcripts was observed in melanoma cell line FON+ along the subcultures.[25,73] This might also have occurred for cultured 4D6 cells as they exhibit a low amount of *HLA-G* transcripts, and HLA-G3 was the only transcript isoform found. A deficit of Aire expression, due to the absence of a specific thymic microenvironment, thus may be hypothesized. Interestingly, wild-type and 4D6 cells transfected with the empty pcDNA3·1 vector were positive for intracellular HLA-G, exclusively located in the perinuclear area forming beam-like structures (Fig. 4a). Therefore, the levels of HLA-G transcripts in these cells are sufficient for the intracellular expression of HLA-G and, in view of the alternative splicing analysis, may correspond to the HLA-G3 isoform (Fig. 2a).

Once Aire was expressed within the thymic cells, we observed changes in *HLA-G* transcription levels and differential splicing of HLA-G primary transcripts leading to the appearance of bands corresponding to at least *HLA-G1* and *HLA-G2/G4* transcripts (Fig. 2a). Notably, it was reported that Aire is located within nuclear speckles, interchromatin granule clusters, enriched of transcription factors, and related to the assembly, modification and storage of processing pre-mRNA.[7] Additionally, Aire was shown to regulate the levels of mature mRNA, promoting its accumulation, stabilization and alternative editing.[7]

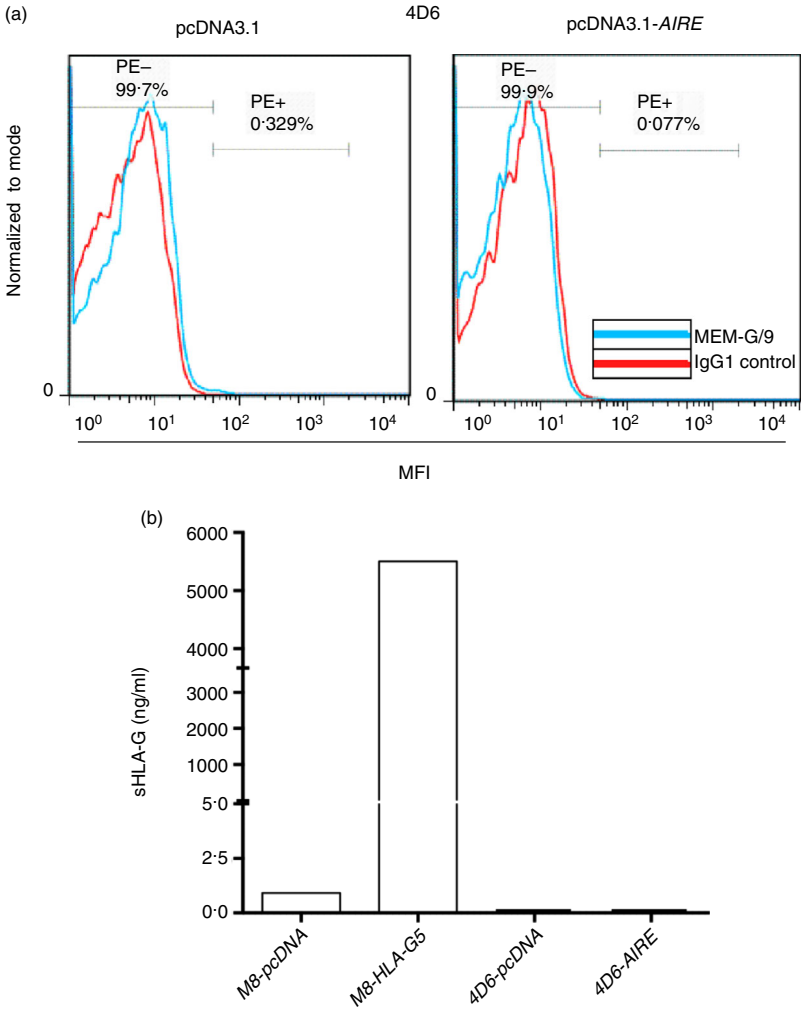

Figure 5. (a) Representative flow cytometry analysis of HLA-G1 cell surface expression on 4D6 human thymic cell line. MFI, mean fluorescence intensity expressed as percentage of anti-HLA-G (MEM-G/9) positive cells relative to the control antibody (anti-IgG1) of at least three independent experiments. (b) ELISA that detects shedding HLA-G1 and HLA-G5 isoforms in culture supernatants (80 μl) using mAb anti-HLA-G MEM-G/9 and anti-β2-microglobulin as capture and detection antibodies, respectively. Culture supernatants of M8 melanoma cell line transfected with HLA-G5 (M8-HLA-G5) or transfected with pcDNA3·1 alone (M8-pcDNA) are the positive and the negative controls, respectively. Culture supernatants of 4D6 human thymic cell line transfected with *AIRE* (4D6-*AIRE*) or not (4D6-pcDNA) were analysed in two independent experiments.

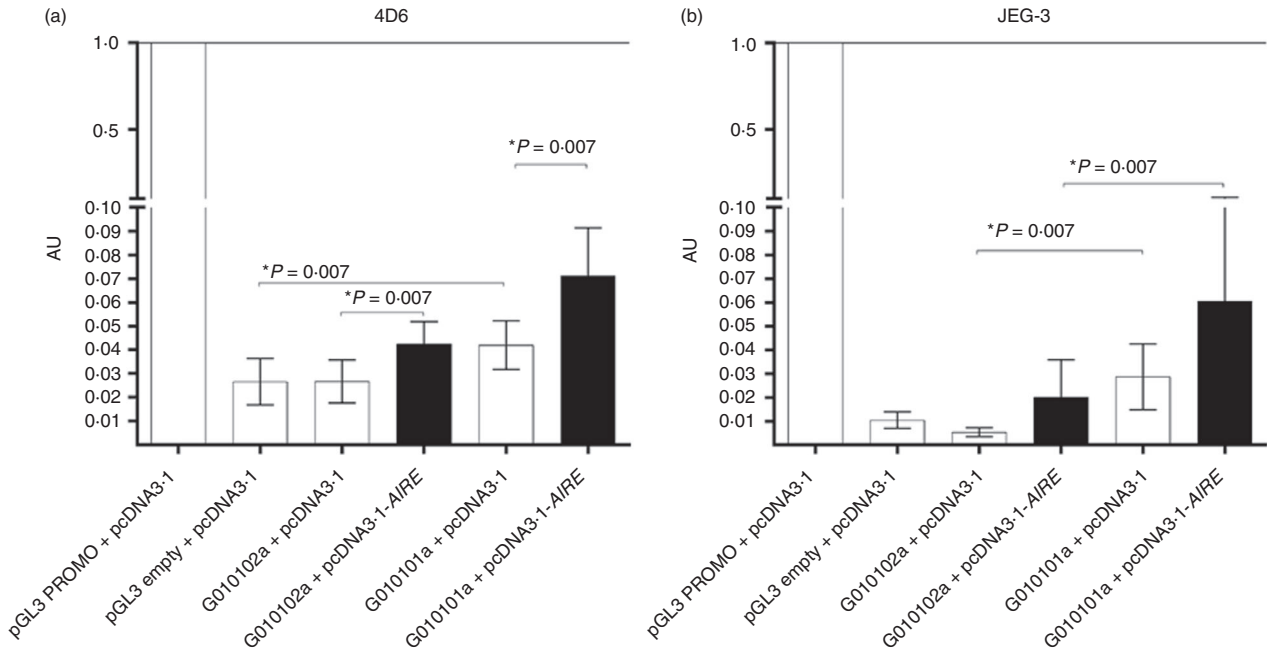

Figure 6. Effect of Aire in the activation of the *HLA-G* 5′-URR in (a) 4D6 and (b) JEG-3 human cell lines. Cells were co-transfected with the two most frequent haplotypes of *HLA-G* gene promoter (*G010101a* and *G010102a*) in the presence of Aire (pcDNA3·1-*AIRE*) or mock plasmid (pcDNA3·1), and subjected to the Dual-Luciferase Reporter activity assays. AU, arbitrary units of luciferase activity. Significance level adopted ($P < 0{\cdot}05$) is represented with (*). Mean of at least five independent experiments. Non-parametric Wilcoxon test was used.

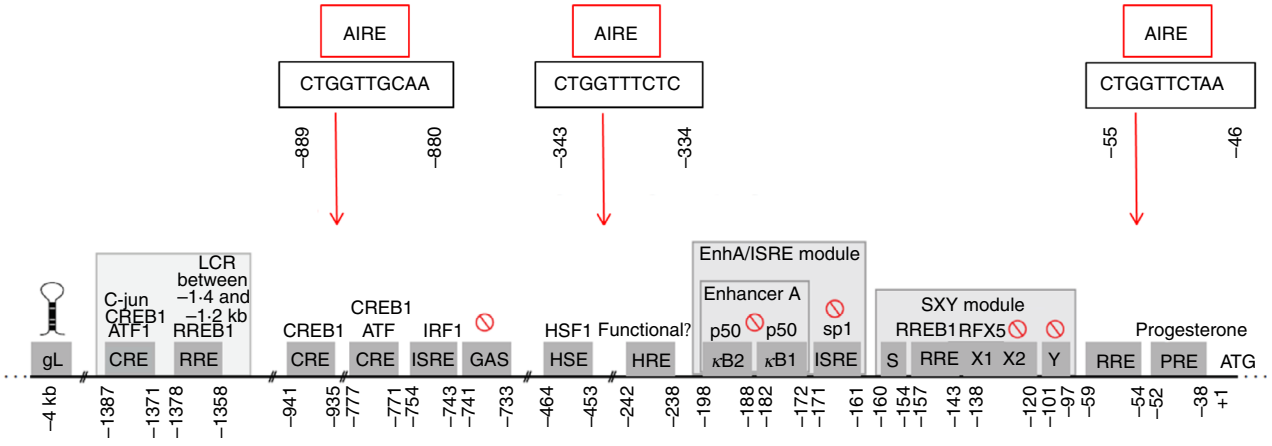

Figure 7. Putative Aire-specific binding sites within the HLA-G 5′-URR. The binding sites sharing the core consensus sequence *CTGGTT* are located at positions -889/-880 (binding site 1 – BS1); -343/-334 (binding site 2 – BS2); and -55/-56 (binding site 3 – BS3). *In silico* analysis was performed with the PROMO platform (*Transfac V6·4*) with a degree of dissimilarity of 15%. Grey boxes localize the main elements involved in the regulation of HLA-G expression (modified picture of previous work published in Ref. [55]). Classical HLA class I regulatory elements that are disrupted in *HLA-G* are indicated by (⃠).

Knowing that HLA-G isoforms G1, G2, G3 and G5 have been described within the medullary compartment of thymus,[31,32] and considering the present study results, it becomes plausible to suggest the involvement of Aire in the alternative splicing of HLA-G in thymic cells. Surprisingly, we observed an overall upregulation of HLA-G protein expression; however, the HLA-G1 isoform was not detected on the cell surface of 4D6 cells. This finding suggests that all isoforms accumulate scattered throughout the intracellular milieu (Fig. 4a) and that additional Aire-independent processing mechanisms may occur in the thymus for HLA-G membrane expression.

By investigating the mechanisms of the Aire-induced upregulation of HLA-G in 4D6 thymic cells, we

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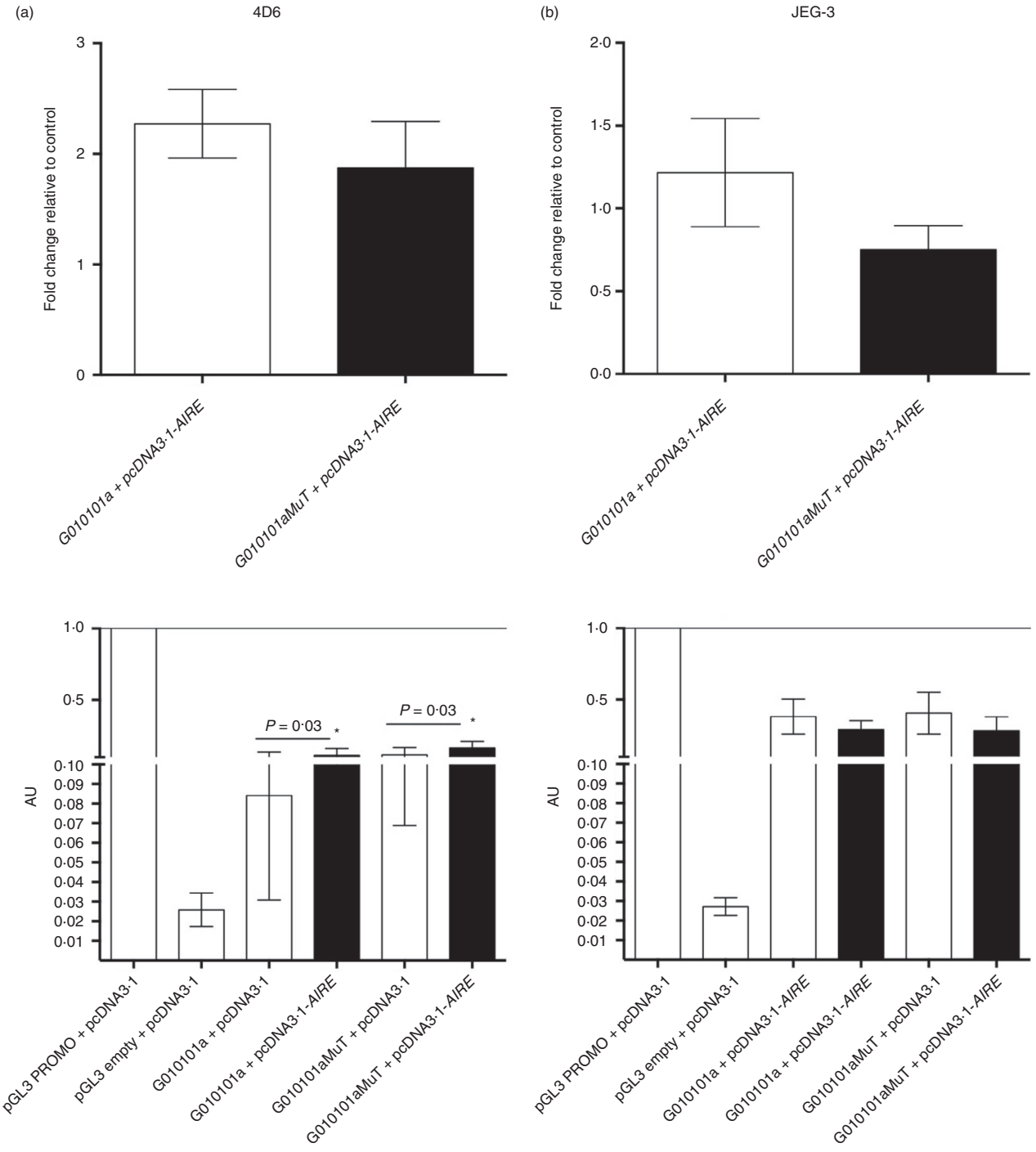

Figure 8. Impact of mutated Aire-specific binding sites on the *HLA-G* 5′-URR activity. Dual-Luciferase Reporter Activity Assays was performed with (a) 4D6 and (b) JEG-3 cell lines. *G010101aWT* represents the most worldwide frequent *HLA-G* 5′-URR haplotype. *G010101aMut* represents the *G010101a* haplotype in which the three putative Aire-binding sites BS1, BS2 and BS3 have been replaced with *AAAAA*. Fold change represents the ratio between the luciferase activities of control-transfected cells with Aire-transfected cells in samples co-transfected with the wild-type and mutated haplotypes. Significance level adopted ($P < 0{\cdot}05$) is represented with (*). Mean of at least five independent experiments). Non-parametric Wilcoxon test was used.

observed that the activity of the two most frequent haplotypes of the 1·4 kb *HLA-G* 5′-URR (*PROMO-G010101a* and *PROMO-G010102a*) was increased in the presence of Aire. Notably, the increase occurred independently of the variable sites, and did not involve the three putative Aire-binding sites (Figs 8 and 9),

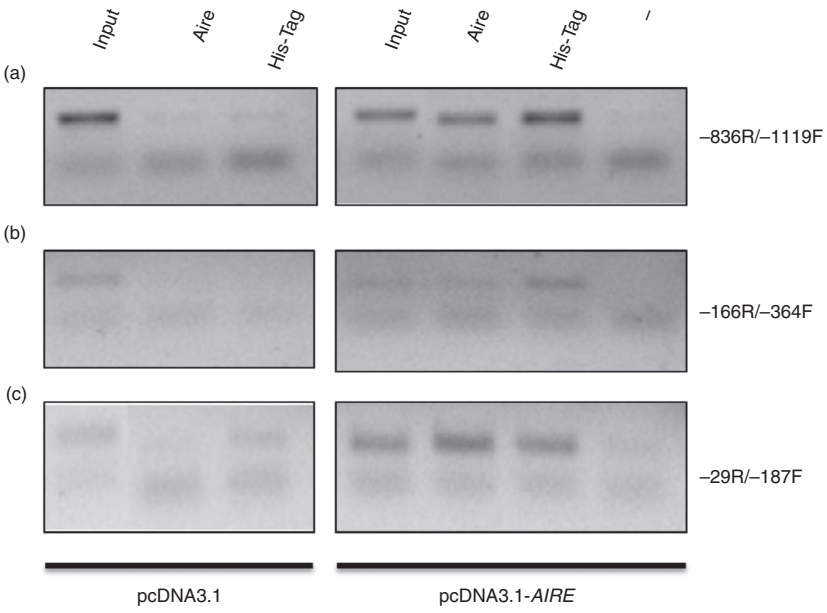

Figure 9. *In situ* binding of Aire to the *HLA-G* 5′-URR. Representative chromatin immunoprecipitation (ChIP) assay carried out with 4D6 human thymic cell line transfected with pcDNA3·1-*AIRE* or mock plasmid (pcDNA3·1) (three independent experiments). (a) 836R/-119F; (b) -166R/-364F and (c) -29R/-187F indicate primer set used for polymerase chain reaction (PCR) targeting BS1, BS2 and BS3 regions of HLA-G 5′-URR, respectively. Input is input chromatin used as PCR control; (-) indicates ChIP performed with IgG control antibody; Aire and His-tag indicate ChIP performed with antibodies directed against Aire and 6 × His-tag, respectively.

respectively. Previous DNA-binding experiments have suggested that Aire preferentially binds to defined DNA *ATTGGTTA* sequence motif;[74] however, in agreement with our result, there is a growing body of evidence that Aire exhibits a low, no-discriminatory affinity for DNA.[75] Interestingly, the main feature of the genes controlled by Aire is that, although they are inactive, they must have the RNAPII previously associated in their promoter regions. This implies that the basal transcription machinery is already engaged, generating only immature, unprocessed and unstable transcripts.[76] At this stage, the C-terminal region of RNAPII is not phosphorylated, preventing the recruitment of the machinery involved in chromatin modification processes.[75,77,78] Indeed, Aire is preferably located in transcriptionally inactive chromatin regions,[77] and the repressive epigenetic signature of Aire-responsive genes includes unmethylated lysine 4 residues in histone H3 (H3K4me0). The H3K4me0 in these genes thus remains unchanged, participating in the recruitment of Aire to the promoter regions via the PHD-1 domain.[77,78] In agreement, we observed that Aire had a significant effect in thymic 4D6 cells, exhibiting low HLA-G transcription, whereas no significant effect was observed with JEG-3 cells, exhibiting high HLA-G transcription. We also showed by ChIP a direct interaction of Aire with *HLA-G* 5′-URR in 4D6 cells, suggesting that, at least, part of the chromatin in this region might exhibit the H3K4me0 repressive mark. Interestingly, Aire is proposed to associate with the transcription start site (TSS) of genes and was detected by ChIP in BS1 and BS3 regions, where a putative alternate HLA-G TSS and a classical one are located, respectively.[79] Otherwise, in cells exhibiting low amounts or absence of HLA-G transcripts, we and other groups previously showed a clear association with repressive epigenetic marks such as histone deacetylation and DNA methylation,[62,63] which could participate in the Aire recruitment.[75,77,78] In addition, Aire binding to DNA–protein complexes was proposed to allow the transient formation of DNA double-strand breaks catalysed by Topoisomerase II that promotes the recruitment of DNA-PK. These events are accompanied by the recruitment of several downstream mediators, such as pTEFb leading to the activation of RNAPII and RNA transcription.[80,81] Accordingly, regarding results obtained with reporter luciferase assay, we can hypothesize that Aire may directly interact with specific partners that bind to the *HLA-G* 5′-URR and, in this way, may drive luciferase reporter activation. On the other hand, it is possible that Aire transfection may have an indirect effect by activating repressed genes that might encode key transcription factors targeting the 1·4 kb *HLA-G* 5′-URR.

All these findings show the ability of Aire in promoting the transcriptional activation of *HLA-G*, specifically in 4D6 thymic cells; the unlike pattern of alternative splicing in the presence of Aire, and also the non-influence of Aire in the transcription of *HLA-E* and *HLA-A* transfected with Aire, lead us to hypothesize that the transcriptional complex of Aire preferably promotes HLA-G expression in thymic microenvironment. HLA-G transactivation by Aire could contribute to the generation of maternal T-lymphocytes that have passed by the thymic selection and are tolerized to HLA-G molecules expressed by fetal cells during pregnancy. The positive expression regulation of HLA-G in the mTECs during the process of PGE regulated by Aire could lead to the generation of clones of HLA-G-tolerized thymocytes, which could

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differentiate into deciduous T-lymphocytes. Thus, during gestation, maternal decidual T-lymphocytes would be irresponsive to fetal tissues, due to the expression of HLA-G on their surface.[82] This model could represent an additional mechanism for the acceptance of the fetus by the maternal organism influenced by the thymus. Furthermore, the HLA-G expression at the cortical-medullary junction could be related to the thymic education events acting for the selection of thymocytes restricted to the recognition of antigens presented by HLA-G. However, this idea is weakened by the fact that a barely reduced repertoire of antigens can bind to the HLA-G cleft, as HLA-G exhibits a low interaction force with the TCR. Thus, the lack of evidence, demonstrating whether the decidual T-cells are restricted to antigens presented by HLA-G or are previously tolerated for HLA-G, lead us to inquire about the biological effect of the presence of HLA-G in the thymus. The results obtained in the present study reinforce the hypothesis that the mechanisms acting on peripheral tolerance, such as the expression of HLA-G, could be controlled by Aire. Thereafter, evidencing a new possible function for Aire on the maintenance of immunological tolerance regulating the mechanisms acting on the peripheral tolerance, through the induction of the expression of immunomodulatory molecules, such as non-classical MHC class I molecules. If true, this phenomenon would represent an evidence of Aire indirectly acting in the periphery, in complementarity to the central tolerance events performed by the thymus.

In conclusion, the present study showed that Aire promotes the transactivation of the *HLA-G* gene by increasing the overall transcription, i.e. inducing the transcription of at least the G1 and G2/G4 isoforms, and incrementing the occurrence and distribution of intracellular HLA-G protein. Aire was also demonstrated to act through the *HLA-G* 5′-URR. The present work thus is a first contribution to the understanding of the mechanisms of the regulation of HLA-G expression in the thymus as well as the enlightenment of its possible biological function and interaction with Aire to the maintenance of central and peripheral tolerance.

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Disclosure

The authors declare that there is no conflict of interests regarding the publication of this article.

Author contributions

BLML designed and performed experiments, analysed data and wrote the paper; IP performed experiments and analysed data; GAP and EDC reviewed the paper; EAD wrote and reviewed the paper; PM designed the experiments, analysed data, wrote and reviewed the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Functional validation of AIRE protein expression in 4D6 and JEG-3 cell lines after transient transfection with pcDNA3·1 or pcDNA3·1-*AIRE*. The Aire expression following transfection of pcDNA3·1-*AIRE* (or empty pcDNA3·1-control vector) in 4D6 and JEG-3 human cell lines was validated by Western blotting using anti-Aire-1 and anti-α-tubulin (mouse mAb IgG1) as internal control. The total hepatocyte cell lysate (Hep G2 cell lysate) was used as a positive control.

Figure S2. Representative RT-PCR analysis showing the simultaneous induction of an Aire-dependent gene (*KERATIN 14*) and *AIRE* in *AIRE*-transfected 4D6 human thymic cell line transfected with AIRE or not. Amplifications were performed on cDNA templates using *AIRE*- and *KERATIN 14*-specific primer sets, and PCR products were separated by electrophoresis in a 1·5% BET-coloured agarose gel. *β*-Actin indicates RT-PCR products used as internal controls. The side graph indicates the quantification of the intensity of BET staining bands area for KERATIN 14 PCR products captured by the Chemidoc Image system (Biorad) and analysed by Image lab software (Biorad, version 5·1 beta). AU = Arbitrary Units: quantification of the intensity of staining bands from KERATIN 14 PCR products normalized by the intensity of β-actin PCR products in transfected 4D6 cells.