

# Macrophages overexpressing Aire induce CD4<sup>+</sup>Foxp3<sup>+</sup> T cells

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**Abstract.** Aire plays an important role in central immune tolerance by regulating the transcription of thousands of genes. However, the role of Aire in the peripheral immune system is poorly understood. Regulatory T (Treg) cells are considered essential for the maintenance of peripheral tolerance, but the effect of Aire on Treg cells in the peripheral immune system is currently unknown. In this study, we investigated the effects of macrophages overexpressing Aire on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells by co-culturing Aire-overexpressing RAW264.7 cells or their supernatant with splenocytes. The results show that macrophages overexpressing Aire enhanced the expression of Foxp3 mRNA and induced different subsets of Treg cells in splenocytes through cell-cell contact or a co-culture supernatants. TGF- $\beta$  is a key molecule in the increases of CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cell and activating Treg (aTreg) levels observed following cell-supernatant co-culturing. Subsets of Treg cells were induced by Aire-overexpressing macrophages, and the manipulation of Treg cells by the targeting of Aire may provide a method for the treatment of inflammatory or autoimmune diseases.

## Introduction

The autoimmune regulator, Aire, was first cloned by German scientists in 1997. Aire is composed of four domains (LXXLL, PHD, SAND and HSR) that are characteristic of transcription factors (1,2), suggesting that Aire may also act as a transcription factor. The mutation or deletion of Aire in humans results in a severe autoimmune disease, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED); Aire is thus considered to play a critical role in self-tolerance. Aire is highly expressed in medullary thymic epithelial cells (mTECs), where it leads to the elimination of autoreactive T cells by regulating the expression of thousands of tissue-restricted anti-

gens (TRAs), which are presented to thymic T lymphocytes by mTECs or cross-presented by dendritic cells (DCs).

Aire is also expressed in the peripheral immune organs and tissues, especially in peripheral blood and the lymph nodes. The two cell types that express Aire in the periphery are myeloid, including DCs and macrophages (3,4), and stromal, including epithelial cells (5,6). Although the cell types that express Aire are known, the function of Aire at these sites remains undefined. Ramsey *et al* proposed that Aire may affect the maturation and antigen presentation of DCs (7). Fletcher *et al* reported that Aire expressed in the stromal cells of the lymph nodes may be involved in peripheral immune tolerance by regulating TRAs in a manner complementary to its role in mTECs (6). However, whether Aire also affects peripheral tolerance by other mechanisms remains ambiguous.

CD4<sup>+</sup> regulatory T (Treg) cells were first identified by Sakaguchi *et al* in 1995 with the observation that depletion of these cells in mice leads to autoimmunity in multiple organs (8). Treg cells represent an essential cell population in the maintenance of peripheral immune tolerance. Miyara *et al* classified CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells into three subsets, rTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup>, resting Treg), aTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup>, activating Treg) and non-Treg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup>) (9). rTreg and aTreg cells have a suppressive function, while non-Treg cells do not suppress effector T cells, but rather secrete a certain level of IL-17.

In the current study, in order to determine whether the expression of Aire in the peripheral immune system influenced Treg cells, the effects of macrophages overexpressing Aire on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and Treg subsets were detected by co-culturing Aire-overexpressing RAW264.7 cells or their supernatant with splenocytes.

## Materials and methods

**Cell culture and mice.** RAW264.7 cells were obtained from the Shanghai Cell Research Institute. The RAW264.7 cells were transfected with pEGFPC1/Aire or pEGFPC1 plasmids and stable cell lines were obtained following G418 selection (10). The cells were cultured in 10% NCS-RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA). Balb/c mice were purchased from the Experimental Animal Center of Jilin University and kept under pathogen-free conditions. All animal procedures were approved by the Ethics Committee of Jilin University.

**Co-culture of the RAW264.7 cell lines with mouse splenocytes.** The spleens were removed from the Balb/c mice and gently

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dissociated into single-cell suspensions followed by ammonium chloride lysis to remove red blood cells. RAW264.7 cells overexpressing Aire (Aire cells) or empty vector (control cells) were seeded onto 96-well plates at  $1 \times 10^5$  per well in 100  $\mu$ l. When the cells had attached to the dishes, they were co-cultured with  $1 \times 10^6$  freshly isolated splenocytes for 48 and 72 h. In addition, 100  $\mu$ l supernatants from the Aire or control cells were co-cultured with an equal number of splenocytes for 48 and 72 h. Afterwards, the suspended splenocytes were harvested for analysis by flow cytometry and real-time PCR.

**RNA isolation and quantitative real-time PCR.** Total RNA was extracted from the harvested splenocytes (as described above) or from the RAW264.7 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and dissolved in DEPC-treated water. The amount of total RNA was measured with an ultraviolet spectrophotometer. A 1- $\mu$ g sample of RNA was used to synthesize cDNA with random primers and AMV reverse transcriptase (Takara, Shiga, Japan) using the following reaction parameters: 30°C for 10 min, 45°C for 30 min and 5°C for 5 min for one cycle. cDNA was used for real-time PCR with the following primers: GAPDH sense, 5'-GAC TTC AAC AGC AAC TCC CAC TC-3' and antisense, 5'-TAG CCG TAT TCA TTG TCA TAC CAG-3'; and Foxp3 sense, 5'-TAC TCG CAT GTT CGC CTA CTT CA-3' and antisense, 5'-ATT CAT CTA CGG TCC ACA CTG CT-3'. The reaction parameters used were as follows: 95°C for 30 sec, 95°C for 5 sec, and 60°C for 30 sec for 40 cycles. The samples were detected with the ABI7300 real-time PCR instrument and the results were analyzed using the formula  $2^{-\Delta\Delta C_t}$ .

The primers and reaction parameters used in RT-PCR are shown in Table I. ImageMaster VDS (Pharmacia Biotech, Tokyo, Japan) image analysis software was employed to analyze the optical density value, and the mRNA expression levels of various genes were expressed as the gene/ $\beta$ -actin optical density value.

**Western blot analysis.** The cells were lysed on ice in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS and a protease inhibitor cocktail (Roche, Mannheim, Germany). Cellular lysates were centrifuged at 10,000  $\times$  g for 10 min and the supernatants were subjected to western blot analysis with anti-TGF- $\beta$  (R&D Systems, Minneapolis, MN, USA) at 1:250 dilution. Finally, the signal was detected with a GeneGnome Imaging System (Syngene, Cambridge, UK) using BeyoECL Plus (Beyotime Biotech., Jiangsu, China).

**Flow cytometry analysis.** The cells were collected and counted, and  $1 \times 10^6$  cells were suspended in PBS (total volume 100  $\mu$ l). PE-Cy7-anti-CD4 (eBioscience, San Diego, CA, USA) and PE-anti-CD45RA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies were added to the cells which were then incubated at 4°C for 40 min. The cells were then fixed with the Foxp3 fixation/permeabilization concentrate and diluent (eBioscience) for 1 h and then treated with 0.1% saponin (Sigma, St. Louis, MO, USA) and AF647-anti-Foxp3 antibody (eBioscience) at 4°C for 1 h. The cells were then washed with PBS and resuspended in 2% paraformaldehyde for analysis using a BD FACSCalibur flow cytometer.

**ELISA.** A TGF- $\beta$ 1 ELISA (R&D Systems) kit was employed and the assay was performed according to the manufacturer's instructions.

**TGF- $\beta$  blocking.** The supernatants secreted from the Aire or control cells were neutralized with TGF- $\beta$  antibody (R&D Systems) for 1 h. Freshly isolated splenocytes were then added to the supernatants and incubated for 48 and 72 h. The splenocytes were harvested for FACS analysis.

**Statistical analysis.** The percentages of each subset of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells among the CD4<sup>+</sup> T cells were calculated using the following formulae (Fig. 1): i) CD4<sup>+</sup>Foxp3<sup>+</sup>T = (P3 + P4 + P6 + P7)/(P2 + P5); ii) rTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup> T) = P3/(P2 + P5); iii) aTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T) = P7/(P2 + P5); iv) non-Treg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup> T) = P6/(P2 + P5); v) CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T = P4/(P2 + P5).

All data are expressed as the means  $\pm$  SD. Differences were compared between the two groups using Student's t-tests. A value of  $p < 0.05$  was considered to indicate a statistically significant result.

## Results

**Aire upregulates Foxp3 mRNA expression in splenocytes.** To observe the effects of Aire on Treg cells, we measured the expression of Foxp3 mRNA, a specific marker of CD4<sup>+</sup> Treg cells that is critical in the development and suppressive function of CD4<sup>+</sup> Treg cells. The Foxp3 mRNA levels were upregulated in the splenocytes co-cultured for 48 h with Aire cells compared with those in the control cells (Fig. 2A). There was a similar change when the splenocytes were co-cultured with the supernatant of the Aire cells for 72 h (Fig. 2B). These results indicate that Treg cells are upregulated by the Aire-overexpressing RAW264.7 cells. It appears that the effect of the Aire cells on Foxp3 expression occurs earlier by cell-cell contact than by contact with the supernatant.

**Aire increases CD4<sup>+</sup>Foxp3<sup>+</sup> T cell production.** The data presented in the previous section show an increase in the mRNA levels of a specific marker of CD4<sup>+</sup> Treg cells. We then detected the number of Treg cells by FACS. The percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells was higher when the spleen cells were co-cultured with Aire cells at 72 h, but not at 48 h. We observed no differences in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells when the splenocytes were co-cultured with the supernatants of Aire or control cells at either 48 or 72 h (Fig. 3). These data reveal that the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells was increased by the Aire cells. Moreover, the Aire cells were able to affect the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells through cell-cell contact, but not through a product present in the supernatant.

**Aire affects the subsets of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells.** The population of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells is composed of three different subsets: rTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup> T cells), aTreg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells) and non-Treg (CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup> T cells) which differ in expression of Foxp3 and CD45RA. To investigate the influence of Aire on these subsets of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, we measured the change in the percentage of each subset after co-culturing with Aire or control cells or

Table I. Primers and reaction parameters for RT-PCR.

Gene	Sequences (5'-3')	Size (bp)	Reaction parameters	Cycles
TGF- $\beta$	S: GCCCTGGATACCAACTATTGC AS: GCAGGAGCGCACAATCATGTT	327	94°C for 2 min, 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, 72°C 10 min	28
$\beta$ -actin	S: TGGAATCCTGTGGCATCCATGAAAC AS: TAAACGCAGCACAGTAACAGTCCG	360	94°C for 2 min, 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, 72°C for 10 min	25

S, sense; AS, antisense.

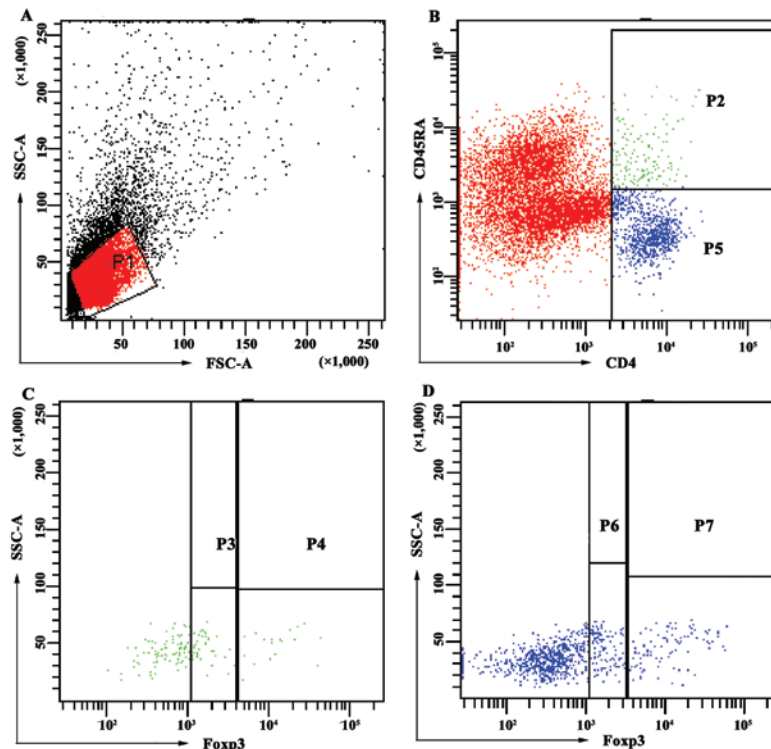


Figure 1. CD4<sup>+</sup>Foxp3<sup>+</sup> T cells were analyzed by flow cytometry. (A) Lymphocytes were gated (P1) and (B) divided into CD4<sup>+</sup>CD45RA<sup>+</sup> and CD4<sup>+</sup>CD45RA<sup>-</sup> T cells (P2 and P5). (C) The CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were divided into CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>lo</sup> and CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells (P3 and P4). (D) The CD4<sup>+</sup>CD45RA<sup>-</sup> T cells were divided into CD4<sup>+</sup>CD45RA<sup>-</sup>Foxp3<sup>lo</sup> and CD4<sup>+</sup>CD45RA<sup>-</sup>Foxp3<sup>hi</sup> T cells (P6 and P7).

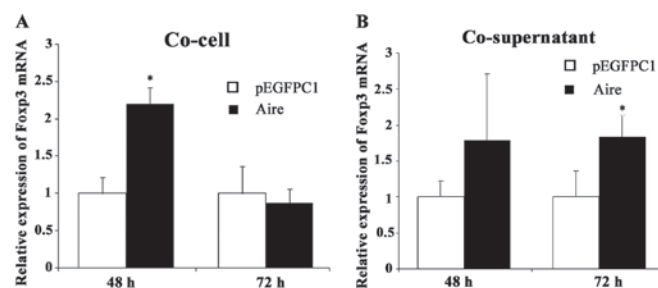


Figure 2. Foxp3 mRNA levels in splenocytes were upregulated when co-cultured with Aire cells. (A) Aire cells, control cells or (B) the supernatants from these cells were co-cultured with freshly isolated spleen cells for 48 and 72 h. The Foxp3 mRNA levels were detected by real-time PCR. Each experiment was repeated at least three times. \*P<0.05 vs. pEGFPC1.

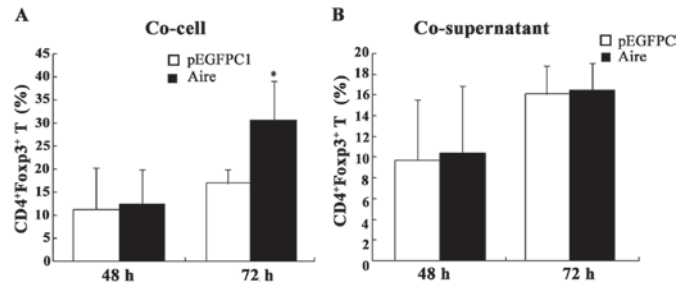


Figure 3. Aire affects the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells. Freshly isolated spleen cells were co-cultured with (A) Aire cells, control cells or (B) the supernatants from these cells for 48 and 72 h. The percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells among the CD4<sup>+</sup> T cells were then detected by FACS. Each experiment was repeated at least three times. \*P<0.05 vs. pEGFPC1.

their supernatants. When the splenocytes and Aire cells were co-cultured for 48 and 72 h, the percentage of rTreg cells

among the CD4<sup>+</sup> T cells decreased at 48 h and significantly increased at 72 h, but the percentages of aTreg and non-Treg

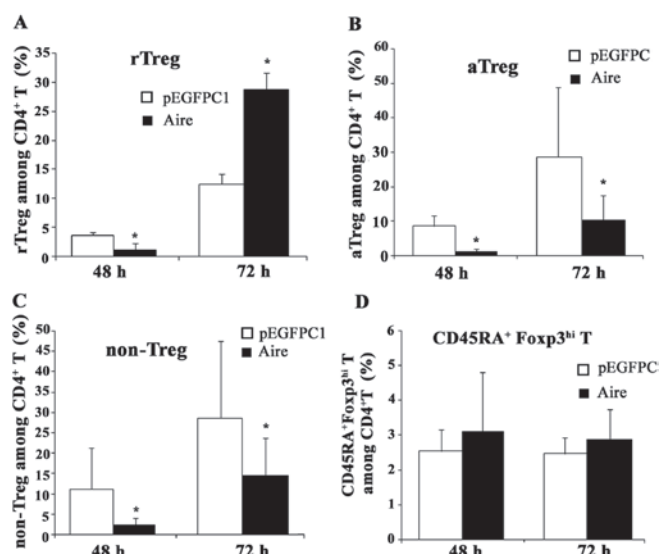


Figure 4. Effects of Aire cells on the numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells and their subsets by cell-cell contact. Spleen cells were co-cultured with Aire or control cells for 48 and 72 h; then the percentages of rTreg, aTreg, non-Treg and CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells among the CD4<sup>+</sup>T cells were analyzed by FACS. Each experiment was repeated at least three times. \*P<0.05 vs. pEGFPC1.

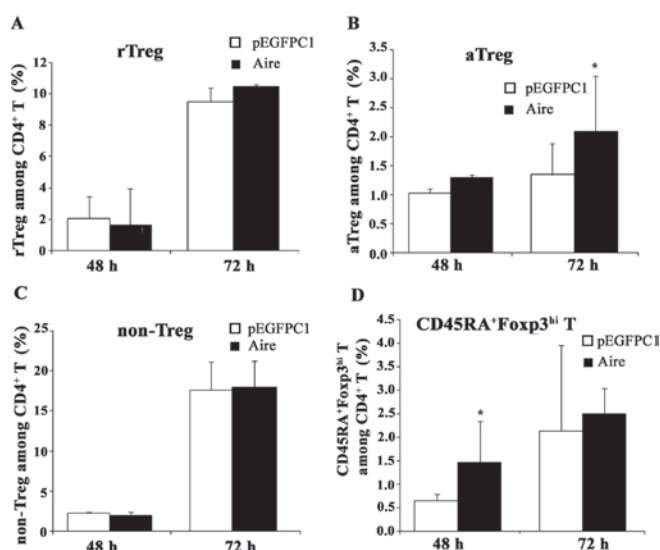


Figure 5. Supernatant of Aire cells affects the numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells and their subsets. Spleen cells were cultured in the presence of supernatants from Aire or control cells for 48 and 72 h, and the percentages of rTreg, aTreg, non-Treg and CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells among the CD4<sup>+</sup>T cells were analyzed by FACS. Each experiment was repeated at least three times. \*P<0.05 vs. pEGFPC1.

cells were lower than in the control cells (Fig. 4). When the spleen cells were cultured with the supernatants secreted from the Aire or control cells, no significant changes were observed in the rTreg and non-Treg cell populations, but the percentage of aTreg cells was higher in the Aire group at 72 h (Fig. 5). These results suggest that Aire stimulates the production and/or induction of rTreg cells and inhibits the formation of aTreg and non-Treg cells through cell-cell contacts. Aire may also promote macrophages to secrete a specific substance that

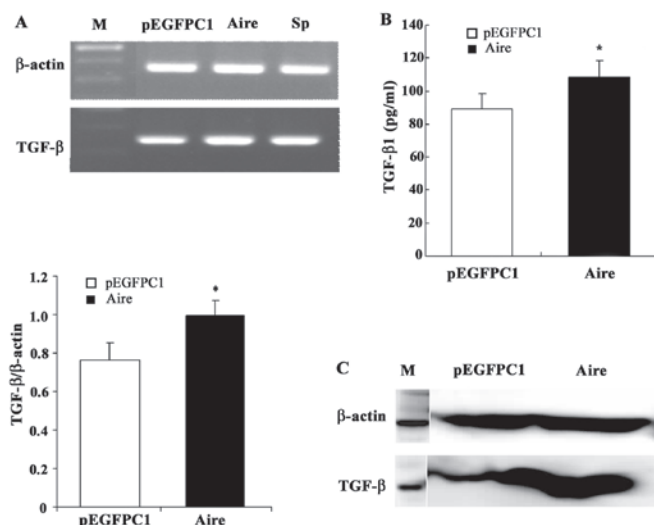


Figure 6. Aire enhances the expression of TGF- $\beta$ . (A) TGF- $\beta$  mRNA levels were measured by RT-PCR. (B) The TGF- $\beta$  concentration in the supernatants was measured by ELISA. (C) TGF- $\beta$  protein levels were measured by western blot analysis. M, marker; pEGFPC1, control cells; Aire, Aire cells; Sp, spleen cells as a positive control. Each experiment was repeated at least three times. \*P<0.05 vs. pEGFPC1.

is able to induce the production of aTreg cells. In addition, we also identified a previously unreported subset of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T, which was also affected by Aire (Figs. 4 and 5), although its function remains unknown. We propose that this population is a transition state from rTreg to aTreg cells and may have a similar function to them.

*TGF- $\beta$  levels are higher in RAW264.7 cells expressing Aire.* As shown in Fig. 5, the supernatants of Aire cells may affect the composition of Treg cells. This suggests that substances in the supernatant may induce the production of Treg cells. Several studies have reported that TGF- $\beta$  induces the production of Treg cells. Therefore, we examined the TGF- $\beta$  levels in the Aire and control cells. The TGF- $\beta$  mRNA and protein levels were higher in the Aire cells than in the control cells (Fig. 6). These data suggest that TGF- $\beta$  is the factor in the supernatant responsible for the induction of Treg cells.

*Anti-TGF- $\beta$  antibody blocks the effects of RAW264.7 cells overexpressing Aire on CD4<sup>+</sup>Foxp3<sup>+</sup> T cells.* To confirm whether TGF- $\beta$  mediates the effects of Aire on CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, an anti-TGF- $\beta$  antibody was used to block the supernatants of the Aire cells prior to the addition of the supernatants to the splenocytes. The expression levels of Foxp3 mRNA were elevated in the splenocytes cultured with the supernatant from the Aire cells at 48 and 72 h. However, when the splenocytes were incubated with the blocked supernatants, the Foxp3 mRNA expression levels decreased (Fig. 7). We also evaluated the effects in each subset of the CD4<sup>+</sup>Foxp3 T cells cultured with supernatants neutralized with the anti-TGF- $\beta$  antibody. The supernatant from the Aire cells increased the percentage of CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells at 48 and 72 h, but this increase did not occur when the supernatant was blocked with anti-TGF- $\beta$  (Fig. 8). The percentage of aTreg cells increased at

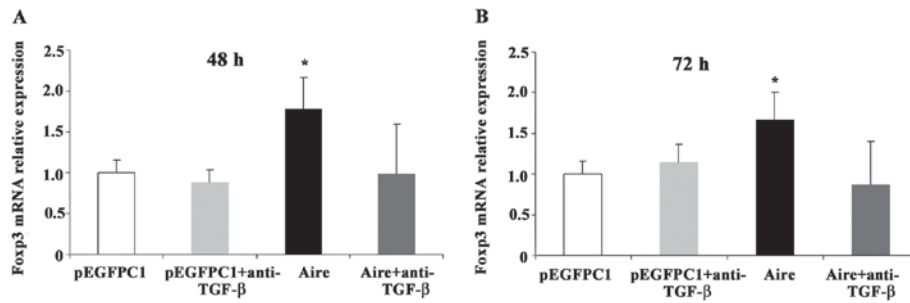


Figure 7. TGF- $\beta$  mediates the upregulation of Foxp3 mRNA by Aire. Foxp3 mRNA expression in splenocytes was examined by real-time PCR after culturing with supernatants that were either untreated or preblocked with anti-TGF- $\beta$  antibody. Each experiment was repeated at least three times. Each group was compared with the pEGFPC1 group. \* $P < 0.05$  vs. pEGFPC1.

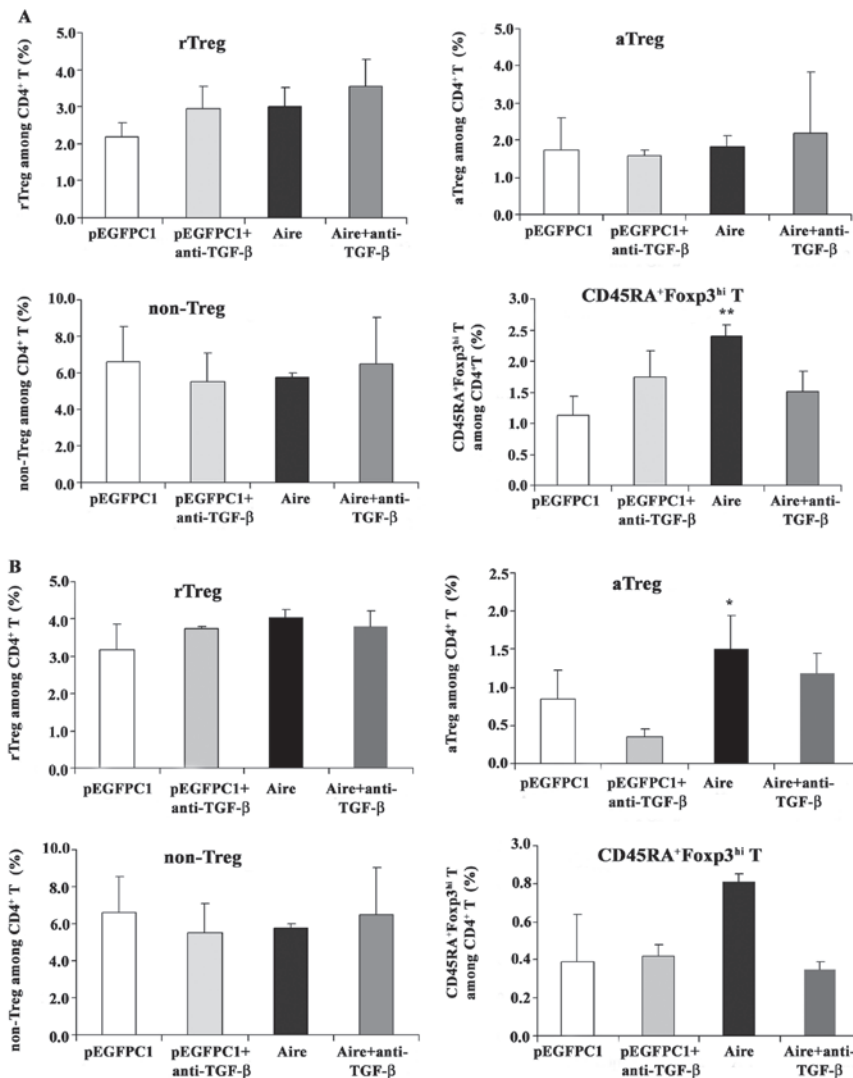


Figure 8. TGF- $\beta$  mediates the effects of Aire on the numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cell subsets through the supernatant. The supernatants of Aire cells or control cells were treated with anti-TGF- $\beta$  antibody (10  $\mu$ g/ml) for 1 h. The spleen cells were cultured with these supernatants for (A) 48 h and (B) 72 h, and the percentages of rTreg, aTreg, non-Treg and CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells among the CD4<sup>+</sup> T cells were analyzed by FACS. Each experiment was repeated at least three times. Each group was compared with the pEGFPC1 group. \* $P < 0.05$  vs. pEGFPC1. \*\* $P < 0.01$  vs. pEGFPC1.

72 h after culturing with the supernatant from the Aire cells, but did not decrease to the same level as that of the control group after treatment with the neutralizing anti-TGF- $\beta$  antibody. From these results, we can conclude that Aire promotes

TGF- $\beta$  production and induces an increase in the number of CD4<sup>+</sup>CD45RA<sup>+</sup>Foxp3<sup>hi</sup> T cells. The induction of aTreg cells following co-culture with Aire cells may be mediated by other molecules besides TGF- $\beta$ .



## Discussion

The role of Aire in the central immune organs, particularly in the thymus, is well defined. Aire functions to eliminate autoreactive lymphocytes through negative selection by regulating the expression of tissue restricted antigens (TRAs). Aire is also thought to affect the positive selection of Treg cells that mediate peripheral immune tolerance. This viewpoint is based on the following observations: the number of Treg cells is lower in APECED patients (11); and T cell receptor (TCR)-influenza hemagglutinin (HA) mice crossed with Aire-HA mice produce more CD4<sup>+</sup>TCR-HA<sup>+</sup>Foxp3<sup>+</sup> Treg cells (12). Aire is also involved in chemokine receptor XCR1 ligand (XCL1)-mediated medullary thymic DC recruitment and contributes to Treg cell production (13). Furthermore, studies of mice with deficiencies in Foxp3 and/or Aire show that mice deficient in the two genes do not have a more severe manifestation than mice deficient in a single gene (14).

Although the functions of Aire in the thymus have been extensively studied, the functions of Aire in the periphery remain unclear and debated. The aim of this study was to examine the extrathymic functions of Aire using Aire-overexpressing RAW264.7 cells.

We found that Aire overexpressed in RAW264.7 cells affected the different subsets of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Through cell-cell contacts, Aire cells were able to promote the production of rTreg cells and inhibit the production of aTreg and non-Treg cells. However, the exact mechanisms by which Aire affects Treg cells are not clear. A recent study reported that some fungi induced the production of Treg cells through TLR3 (15), and we previously reported that Aire promotes the expression of TLR3 (10). A pathway mediated through TLR3 is likely to be one of the mechanisms by which Treg cells are induced. A deficiency in Aire may affect the expression of TLR3 and therefore result in autoimmune disease.

TGF- $\beta$  is generally considered to be a potent inducer of Treg cells (16). Given that Aire overexpression upregulates TGF- $\beta$  expression in macrophages and induces aTreg production, Aire may also enhance the secretion of TGF- $\beta$  to induce the production of aTreg cells to suppress immune responses and prevent autoimmune disease. In addition, based on previous reports, IL-6 potently prevents the TGF- $\beta$ -mediated development of Treg cell induction and instead acts with TGF- $\beta$  to induce Th17 cell differentiation (17). Stimulation of Treg cells in the presence of IL-6 results in loss of Foxp3 expression and acquisition of a Th17 cell phenotype and function (18,19). In our previous study, although IL-6 expression in RAW264.7 cells overexpressing Aire increased following treatment with LPS, it was significantly lower than in the LPS-treated control cells (unpublished data). This means that the expression of Aire may inhibit IL-6 expression, thus leading to the induction of Treg cell production and suppression of Th17 cell production.

Patients with a mutation in Aire (APECED) often contract a cutaneous *Candida albicans* infection in addition to autoimmune diseases. As previously mentioned, overexpression of Aire in macrophages enhances TLR3 expression and promotes the production of Treg cells. In addition, *C. albicans* is one of the fungi that induces Treg cells via TLR3 (15). This result may provide an explanation for the manifestation of *C. albicans*

in Aire-deficient patients. A recent study showed increased secretion of IL-17A in response to *C. albicans* in patients with autoimmune polyendocrine syndrome type 1 (20). Non-Treg cells have the ability to produce IL-17 and overexpression of Aire in macrophages inhibits the production of non-Treg. This suggests that APECED patients, who are deficient in Aire, may not be able to suppress the overproliferation and activation of non-Treg cells that then produce a large amount of IL-17 and mediate the response to *C. albicans*.

In conclusion, we found that the overexpression of Aire in RAW264.7 cells may affect the induction of different subsets of Treg cells. This finding yields a possible explanation for the prevalence of *C. albicans* infections in patients with autoimmune polyendocrine syndrome type 1 and may also lead to new therapeutics for the treatment of autoimmune disease and transplantation.

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