

Original Article

Autoimmune regulator affects macrophage polarization in murine RAW 264.7 cells

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Abstract: The role of autoimmune regulator (AIRE) in the peripheral tolerance is poorly understood. We hypothesize that AIRE may affect peripheral immune tolerance, to a certain extent, which causes the susceptibility to *Candida* infection in APS I patients. The GFP/RAW cells and GFP-AIRE/RAW cells were cultured. After M1 ELISA induction, NO in the cell supernatant was detected. The intracellular Arg1 activity was assessed. Our results showed AIRE could promote classically activated macrophages (M1) polarization, up-regulate inflammatory cytokines such as IL-1 α , and nitric oxide (NO) mRNA and protein expression. Meanwhile, AIRE inhibited alternatively activated macrophages (M2) polarization, down-regulated anti-inflammatory cytokines such as Arg1, IL-10, and GAS6 mRNA and protein expression. In addition, AIRE mRNA expression was closely related to the macrophages polarization, and it was up-regulated when RAW 264.7 cells were induced to M1 phenotype and down-regulated when they were in M2 phenotype. Thus, our findings suggest that AIRE may promote macrophage differentiation into M1 phenotype, and enhance the killing of pathogenic microorganisms of macrophages, playing an important role in the peripheral immune tolerance.

Keywords: Autoimmune regulator, autoimmune polyglandular syndrome type I, polarization, RAW 264.7 cells

Introduction

Autoimmune polyglandular syndrome I (autoimmune polyglandular syndrome type I, APS I), also known as Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy Phenotype (APECED) is a rare autosomal dominant autoimmune disease caused by AIRE gene mutation [1]. APS I patients mainly present parathyroid dysfunction, Addison's disease, and *Candida* infection due to immunodeficiency. AIRE in thymic medullary epithelial cells (mTECs) can increase the expression of tissues special antigen (TSA) in peripheral tissues, which may maintain the negative selection of cells by removing autoreactive T cells. Therefore, AIRE plays a key role in the central tolerance. In the peripheral lymphoid organs of secondary lymph nodes, spleen and peripheral blood cells, AIRE is mainly expressed in cells in the lymph node, splenic stromal cells, DC cells, monocytes and macrophages, but far below the amount of mTECs [2, 3]. The specific mechanism underlying the susceptibility to *Candida* APS I patients

are currently unclear. Clinical manifestations and pathologic of APS I may be related to the changes in peripheral immune tolerance, because AIRE plays an important role in maintaining peripheral immune tolerance [4]. AIRE and DNA protein kinase (DNA-PK) synergize TLR1, TLR3 and TLR8 promoter to increase the expression of these genes, thus prompting macrophage-pathogen recognition [5].

Macrophages can rapidly response to infection and injury. Activated macrophages can differentiation into two distinct subgroups: M1 and M2. In the early immune response, M1 macrophages can secrete a variety of inflammatory mediators, such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) and nitric oxide (NO), which may kill pathogens and tumor cells [6]. M2 macrophages, also known as alternative activated macrophages (AAM), can inhibit the development of inflammation. AAM can be further divided into three subtypes: M2a, M2b, M2c. M2a can secrete a large amount of arginine synthase (Arginase, Arg1), which catalyzes the

Table 1. Primers used in real-time RT-PCR

Genes	Primers	Length (bp)
GAPDH	5'-GACTTCAACAGCAACTCCCACTC-3' 5'-TAGCCGTATTTCATTGTCATACCAG-3'	107
AIRE	5'-GAGTCAGGAAGATCCAAGAAGTG-3' 5'-TGCTCATCTCTACCAGGTATAGTG-3'	170
IL-1 α	5'-ACAGTTCTGCCATTGACCATCTC-3' 5'-CTTCAGAATCTTCCCGTTGCTTG-3'	194
iNOS	5'-TGGAGCGAGTTGTGGATTGTC-3' 5'-GGTCGTAATGTCAGGAAGTAG-3'	146
Arg1	5'-GCATATCTGCCAAAGACATCGT-3' 5'-CAATCCCAGCTTGTCTACTTCA-3'	123
IL-10	5'-GCTCTTACTGACTGGCATGAGGA-3' 5'-AGGAGTCGGTTAGCAGTATGTTG-3'	179

polyamide products, to promote the synthesis of collagen, leading to tissue remodeling [7]. M2b is a special subtype of M2, and secret a small amount of inflammatory cytokine IL-12 and a large amount of anti-inflammatory cytokine IL-10 [8]. M2c is characterized by the secretion of a large amount of cytokines such as IL-10 and growth factor β (TGF- β), Growth Arrest Specific Protein 6 (GAS6), and can also promote matrix deposition, causing tissue remodeling [9].

Therefore, we hypothesize that AIRE may affect peripheral immune tolerance, to a certain extent, which causes the susceptibility to Candida infection in APS I patients.

Materials and methods

Secondary screening of RAW 264.7 cells with stable expression of GFP and GFP-AIRE

The GFP/RAW cells and GFP-AIRE/RAW cells were provided by the Department of Immunology, Norman Bethune College of Medicine, Jilin University [10], and maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS; GBICO) plus G418 (AMRESCO) (200 μ g/ml) in 24-well plates. Monoclonal GFP/RAW cells and GFP-AIRE/RAW cells were detected under a fluorescence microscope, and then transferred into 6-well plates, followed by incubation at 37°C in an environment with 5% CO₂.

Preparation of polarized macrophage subsets

The GFP/RAW cells and GFP-AIRE/RAW cells were grown in 6-well plates at 2 \times 10⁶/well over-

night. For induction of macrophage polarization, GFP/RAW cells and GFP-AIRE/RAW cells were maintained in serum-free RPMI 1640. Containing following mediators: IFN- γ (100 ng/ml, peprotech) plus LPS (0.5 μ g/ml, Sigma-Aldrich) was used for 24 hours to induce M1 phenotype; IL-4 (20 ng/ml, R&D) was used to induce M2a phenotype; immune complexes (150 μ g/ml egg albumin monoclonal antibody and 15 μ g/ml chicken egg white protein, Sigma-Aldrich) was used at 37°C in a water bath for 30 min combine with LPS for 24 hours to induce M2b phenotype; IL-10 (20 ng/ml, R&D) was used for 24-h to induce M2c phenotype.

RNA extraction and reverse transcription

Cells were lysed in Trizol reagent (TaKaRa) for the extraction of total RNA. RNA concentration and quality were measured by NanoDrop2000. The RNA concentration in each well, was adjusted to 1000 ng/ μ l, and total RNA was used for reverse transcription into to cDNA, according to the manufacturer's instructions (Thmeor).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with SYBR Green PCR Master Mix Kit (TaKaRa) in quantitative real time PCR instrument (MJ). Primers are shown in **Table 1**. 25 μ l of mixture was added to each well: SYBR[®] Premix Ex TaqII (12.5 μ l), forward primer (1 μ l) and reverse primer (1 μ l), cDNA template (2 μ l < 100 ng) and ddH₂O (8.5 μ l). The melting curve was used for the calculation of mRNA expression.

ELISA

Contents of IL-1 α , IL-10, and Gas6 levels in the supernatants were measured after 24-h cell incubation in serum-free medium by sandwich ELISA according to standard procedure. The optical density (OD) of each well was measured, and standard curve was delineated. The concentration of each cytokines was calculated on the basis of standard curve. IL-1- α ELISA (WestTang, CHINA), IL-10 ELISA kit (MultiSciences, CHINA) and Gas6 ELISA kit (Ray Biotech, USA) were used for the measurement.

Measurement of NO

The supernatant was collected from each well after M1 ELISA induction. NO in the cell super-

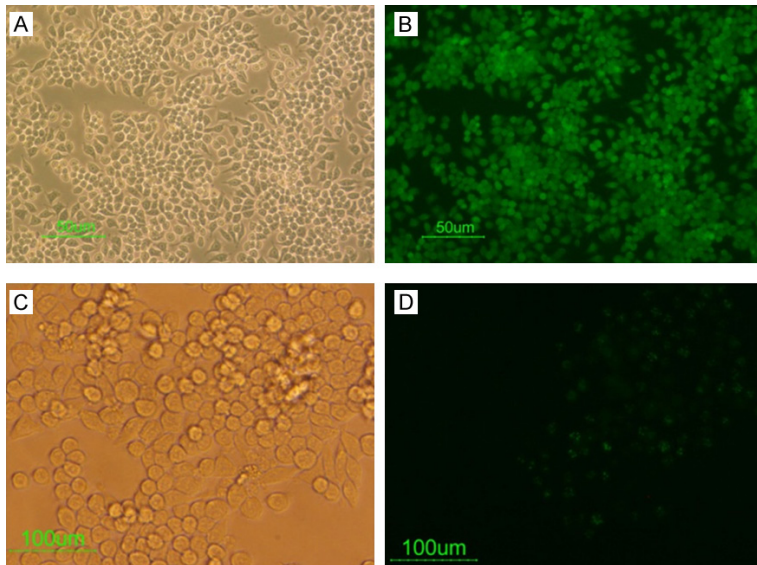


Figure 1. RAW 264.7 cells with stable expression of GFP and GFP-AIRE proteins. (A) GFP/RAW cells under a light microscope; (B) GFP-RAW cells under a fluorescence microscope (A, B: 200×); (C) GFP-AIRE/RAW cells under a light microscope; (D) GFP-AIRE/RAW cells under a fluorescence microscope (C, D: 400×).

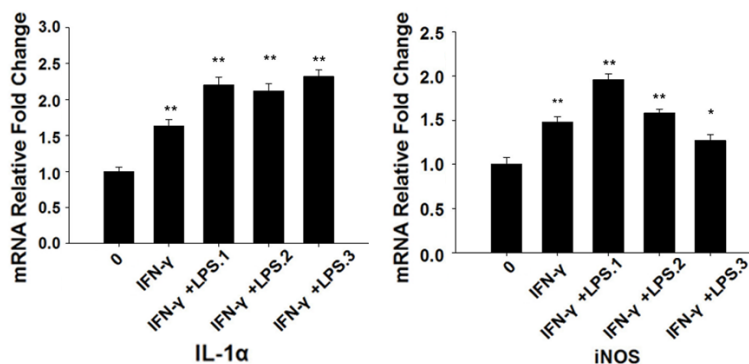


Figure 2. IFN-γ plus LPS could induce RAW 264.7 cells to M1. RAW 264.7 cells treatment with IFN-γ plus LPS at different concentrations could significantly up-regulate the iNOS, IL-1α mRNA expression in RAW 264.7 cells, and IFN-γ at 100 ng/ml plus LPS at 0.5 μg/ml was the optimal concentration. IFN-γ (100 ng/ml), IFN-γ+LPS.1 (100 ng/ml IFN-γ+0.5 μg/ml LPS), IFN-γ+LPS.2 (100 ng/ml IFN-γ+5 μg/ml LPS), IFN-γ+LPS.3 (100 ng/ml IFN-γ+50 μg/ml LPS). Data are expressed as means ± SD from three independent experiments. Treated group vs control group. *P < 0.05, **P < 0.01.

nantant was detected using the Griess Reagent (Beyotime, CHINA). The amount of NO in the supernatant was calculated based on the standard curve.

Measurement of Arg1 activity

The intracellular Arg1 activity was assessed though measuring the total L-arginine metabo-

lites of urea [11]. Urea was measured according to the manufacturer's instructions. (Bioassay Systems, No.DIUR-500, USA), and Arg1 activity was calculated based on the standard curve.

Statistical analysis

All the data from three independent experiments are expressed as mean ± standard deviation (SD). Statistically analysis was performed with SPSS 19.0, and comparisons were done with Student's t test. A value of P < 0.05 was considered statistically significant.

Results

Successful secondary screening of RAW 264.7 cells with stable expression of GFP and GFP-AIRE proteins

Cells were maintained in RPMI 1640 medium containing 10% FBS plus G418 (200 μg/ml) in 24-well plates for further resistant screening. Under the fluorescence microscope, a few cells insensitive to G418 were floating on the medium. Meanwhile, some monoclonal cell and were survived adherent to the plate. Under a fluorescence microscope, GFP-AIRE/RAW cells formed a few colonies with fluorescence, and few colonies with strong green fluorescence were formed by GFP/RAW cells. Cells under a fluorescence microscope after incubation in 24-well plates for 5 days, are shown in **Figure 1**.

AIRE promotes M1 polarization

To promote macrophages into M1 phenotype, RAW 264.7 cells were treated with IFN-γ and LPS at different concentrations, iNOS, IL-1α mRNA expression was detected by qPCR. iNOS

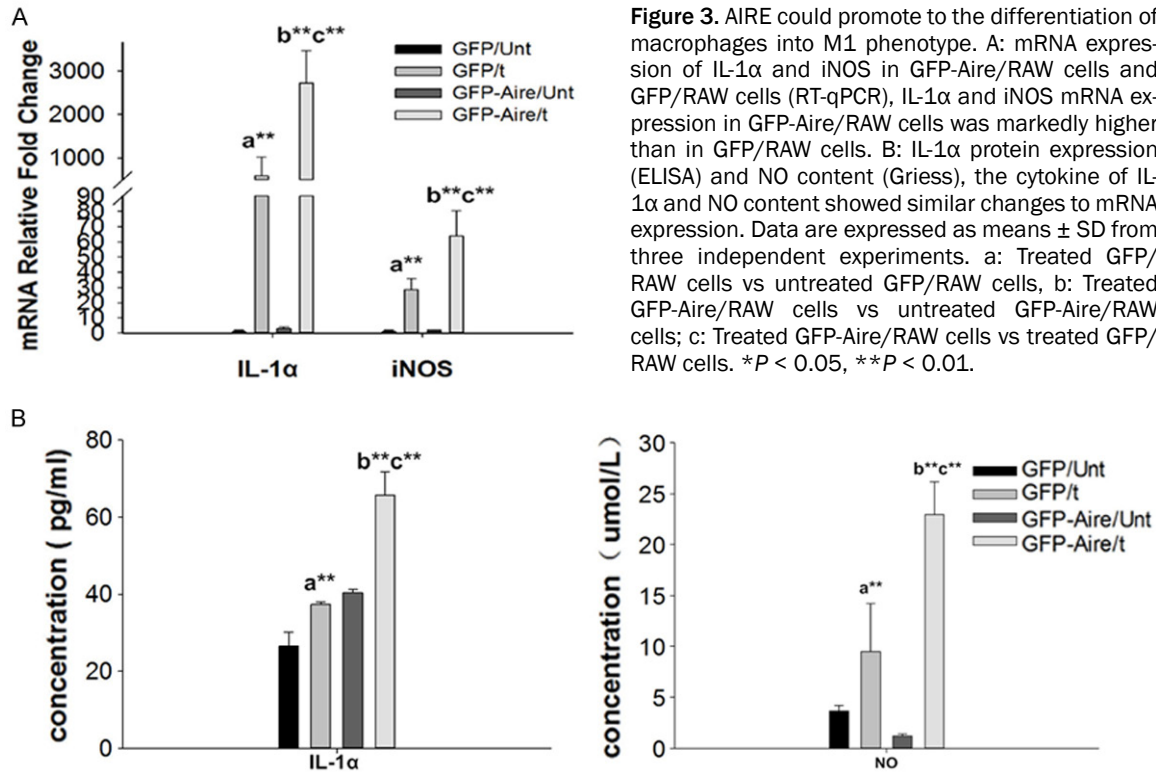


Figure 3. AIRE could promote to the differentiation of macrophages into M1 phenotype. A: mRNA expression of IL-1α and iNOS in GFP-Aire/RAW cells and GFP/RAW cells (RT-qPCR), IL-1α and iNOS mRNA expression in GFP-Aire/RAW cells was markedly higher than in GFP/RAW cells. B: IL-1α protein expression (ELISA) and NO content (Griess), the cytokine of IL-1α and NO content showed similar changes to mRNA expression. Data are expressed as means ± SD from three independent experiments. a: Treated GFP/RAW cells vs untreated GFP/RAW cells, b: Treated GFP-Aire/RAW cells vs untreated GFP-Aire/RAW cells; c: Treated GFP-Aire/RAW cells vs treated GFP/RAW cells. * $P < 0.05$, ** $P < 0.01$.

and IL-1α were markers for M1 phenotype. Results are shown in **Figure 2**. Treatment with IFN-γ plus LPS at different concentrations could significantly up-regulate the iNOS, IL-1α mRNA expression in RAW 264.7 cells, suggesting RAW 264.7 cells were induced to M1 phenotype by IFN-γ and LPS. Moreover, after treatment with IFN-γ at 100 ng/ml plus LPS at 0.5 μg/ml, the iNOS and IL-1α mRNA expression was the highest. Therefore, 100 ng/ml IFN-γ plus 0.5 μg/ml LPS were used for the induction of M1 phenotype in RAW 264.7 cells.

GFP/RAW cells and GFP-Aire/RAW cells were independently treated with IFN-γ (100 ng/ml) plus LPS (0.5 μg/ml) to induce M1 phenotype. After 24-h, total RNA was extracted and the supernatant was collected from different groups. The IL-1α and iNOS mRNA expression was detected by qPCR. Results showed that the IL-1α and iNOS mRNA expression in IFN-γ and LPS treated groups significantly increased as compared to control group. Moreover, after IFN-γ and LPS treatment, the IL-1α and iNOS mRNA expression in GFP-Aire/RAW cells was markedly higher than in GFP/RAW cells (**Figure 3A**). Then, the IL-1α expression and NO content were detected by ELISA, and Griess reaction, respectively. Results showed IL-1α expression

and NO content showed similar changes to mRNA expression (**Figure 3B**). These findings suggest that AIRE may promote to the differentiation of macrophages into M1 phenotype.

AIRE inhibits M2a polarization of macrophages

IL-4 can induce the M2a phenotype of macrophages, which are characterized by elevated expression of Arg1 [7]. In order to investigate whether IL-4 can induce the M2a phenotype of RAW 264.7 cells and the optimal concentration of IL-4. RAW 264.7 cells were treated with IL-4 at different concentrations, and the Arg1 mRNA expression was detected by qPCR. Results are shown in **Figure 4A**. With the increase in IL-4 concentration, Arg1 mRNA expression was up-regulated, and 20 ng/ml was the optimal concentration. The results suggest IL-4 can induce the M2a phenotype of RAW 264.7 cells, and IL-4 at 20 ng/ml is the best treatment for the induction of M2a phenotype of RAW 264.7 cells.

GFP/RAW cells and GFP-Aire/RAW cells were independently treated with IL-4 for 24-h, and total RNA was extracted from each group. Then, Arg1 mRNA expression was detected by qPCR. Results are shown in **Figure 5A**. Results

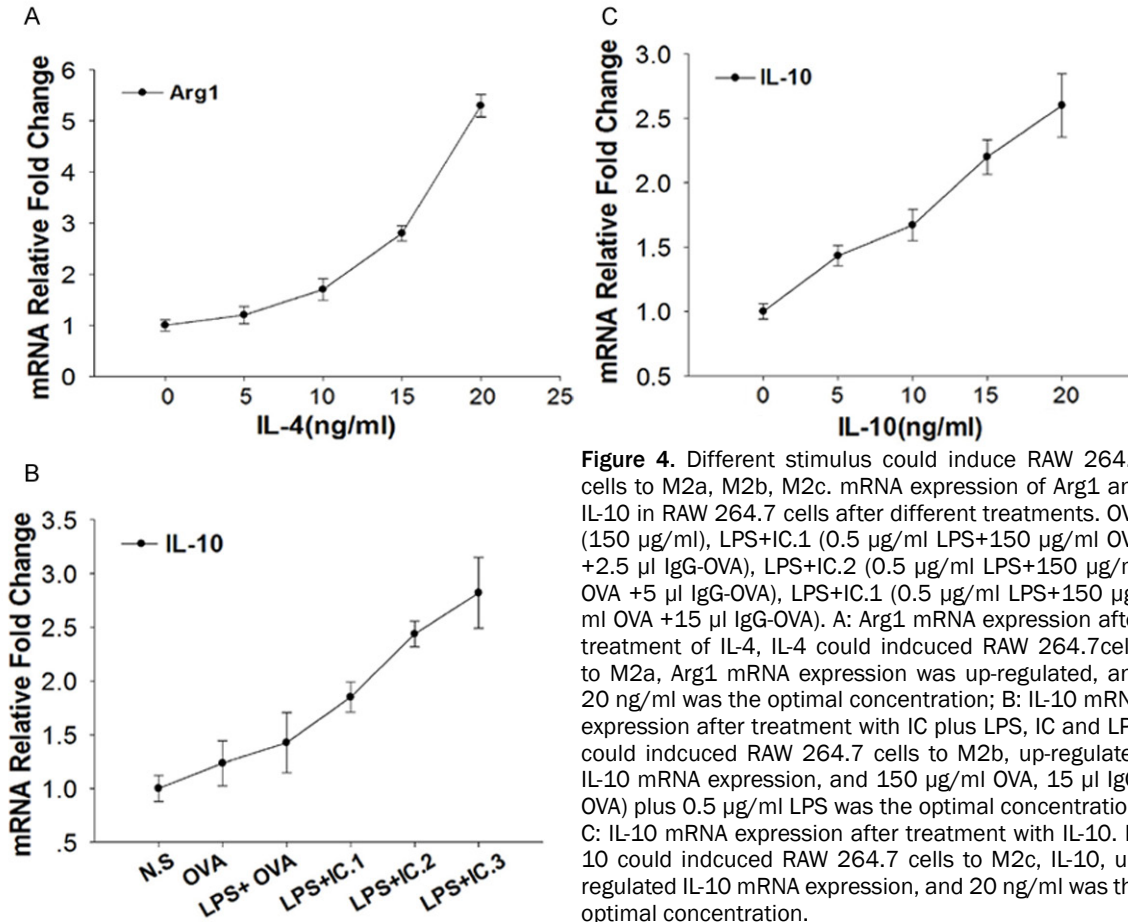


Figure 4. Different stimulus could induce RAW 264.7 cells to M2a, M2b, M2c. mRNA expression of Arg1 and IL-10 in RAW 264.7 cells after different treatments. OVA (150 µg/ml), LPS+IC.1 (0.5 µg/ml LPS+150 µg/ml OVA +2.5 µl IgG-OVA), LPS+IC.2 (0.5 µg/ml LPS+150 µg/ml OVA +5 µl IgG-OVA), LPS+IC.1 (0.5 µg/ml LPS+150 µg/ml OVA +15 µl IgG-OVA). A: Arg1 mRNA expression after treatment of IL-4, IL-4 could induced RAW 264.7 cells to M2a, Arg1 mRNA expression was up-regulated, and 20 ng/ml was the optimal concentration; B: IL-10 mRNA expression after treatment with IC plus LPS, IC and LPS could induced RAW 264.7 cells to M2b, up-regulated IL-10 mRNA expression, and 150 µg/ml OVA, 15 µl IgG-OVA plus 0.5 µg/ml LPS was the optimal concentration; C: IL-10 mRNA expression after treatment with IL-10. IL-10 could induced RAW 264.7 cells to M2c, IL-10, up-regulated IL-10 mRNA expression, and 20 ng/ml was the optimal concentration.

showed Arg1 mRNA expression significantly increased after IL-4 treatment, as compared to control group. Arg1 mRNA expression in GFP-AIRE/RAW cells after IL-4 treatment reduced significantly when compared with GFP/RAW. Furthermore, the Arg1 activity was detected by measuring concentration of urea produced by L-arginine. As shown in **Figure 5B**, the Arg1 activity showed the similar change to Arg1 mRNA expression. These suggest that AIRE may inhibit the differentiation of macrophages into M2a phenotype.

AIRE inhibits macrophage to polarization into M2b and M2c

The M2b macrophages and M2c macrophages have some similarities in their functions. Both are able to secrete a large amount of IL-10, and thus also known as regulatory macrophages. Studies have shown that immune complexes combination with TLR agonist may induce the M2b phenotype of macrophages [13]. In order

to investigate whether immune complexes and TLR agonist can induce the M2b phenotype of RAW 264.7 cells and their optimal concentrations, egg albumin monoclonal antibody and chicken egg white protein at two different concentrations plus LPS (0.5 µg/ml) were used to treat RAW 264.7 cells. Then, IL-10 mRNA expression was detected by qPCR. Results are shown in **Figure 4B**. Results showed treatment with immune complexes and TLR agonist can up-regulated IL-10 mRNA expression, which was the highest after treatment with 150 µg/ml OVA, 15 µl IgG-OVA, and 0.5 µg/ml LPS. These results suggest that the immune complex plus LPS can induce the M2b phenotype of RAW 264.7 cells.

GFP/RAW cells and GFP-Aire/RAW cells were independently treated with immune complexes (150 µg/ml OVA +15µl IgG-OVA) plus LPS (0.5µg/ml), to induce M2b phenotype of macrophages. After 24-h treatment, total RNAs was extracted and the supernatant was col-

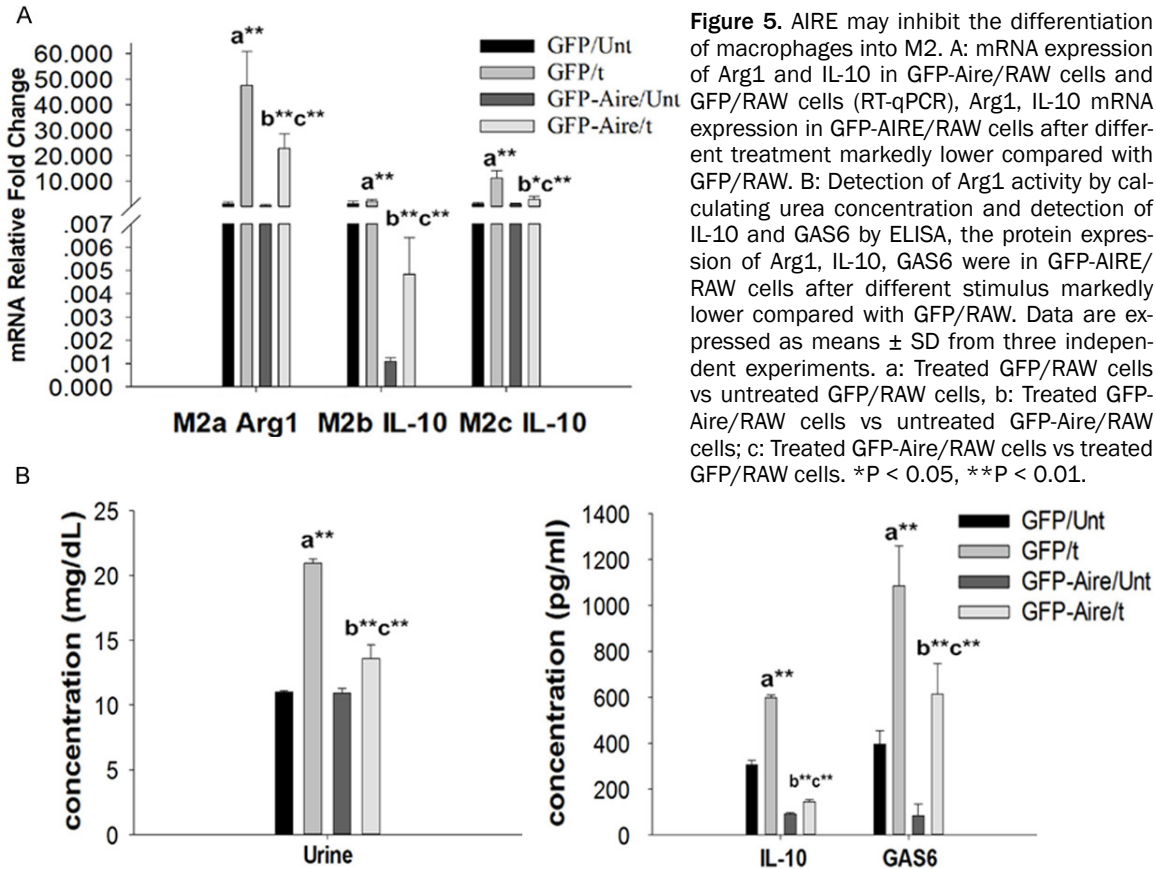


Figure 5. AIRE may inhibit the differentiation of macrophages into M2. A: mRNA expression of Arg1 and IL-10 in GFP-Aire/RAW cells and GFP/RAW cells (RT-qPCR), Arg1, IL-10 mRNA expression in GFP-AIRE/RAW cells after different treatment markedly lower compared with GFP/RAW. B: Detection of Arg1 activity by calculating urea concentration and detection of IL-10 and GAS6 by ELISA, the protein expression of Arg1, IL-10, GAS6 were in GFP-AIRE/RAW cells after different stimulus markedly lower compared with GFP/RAW. Data are expressed as means \pm SD from three independent experiments. a: Treated GFP/RAW cells vs untreated GFP/RAW cells, b: Treated GFP-Aire/RAW cells vs untreated GFP-Aire/RAW cells; c: Treated GFP-Aire/RAW cells vs treated GFP/RAW cells. * $P < 0.05$, ** $P < 0.01$.

lected from each group. The IL-10 mRNA and protein expression was detected by qPCR and ELISA respectively. Results are shown in **Figure 5**. The expression of IL-10 mRNA and protein were up-regulated after treatment with immune complexes + LPS, but that in GFP-AIRE/RAW cells was significantly lower than in GFP/RAW cells. These findings suggest that AIRE may inhibit the M2b phenotype of macrophages.

IL-10 can induce the M2c phenotype of macrophages [9]. In order to investigate whether IL-10 can induce the M2c phenotype of RAW 264.7 cells and the optimal concentration of IL-10, RAW 264.7 cells were treated with different concentrations of IL-10, and then the IL-10 mRNA expression was detected by qPCR. Results showed IL-10 at different concentrations could promote M2c phenotype of RAW 264.7 cells, and IL-10 (20 ng/ml) was the optimal treatment for the induction of M2c phenotype. GFP/RAW cells and GFP-Aire/RAW cells were independently treated with IL-10 (20 ng/ml) to induce the M2c phenotype, and total RNAs was extracted and supernatant was collected from each group after 24-h treatment

(**Figure 4C**). Results showed the expression of IL-10 mRNA in M2c was similar change to the expression of M2b (**Figure 5A**). The IL-10 in the supernatant of M2c macrophages may interfere with the detection of IL-10 by ELISA. Studies have shown that Growth Arrest Specific Protein 6 (GAS6) is a cytokines secreted by M2c macrophages, and the activation of GAS6/MerTK signaling pathway may affect the secretion of IL-10 by M2c macrophages [14]. Therefore, the protein expression of GAS6 was detected by ELISA to indirectly reflect IL-10 expression in M2c macrophages. Results showed GAS6 expression was significantly increased after treatment when compare with control group, and GFP-AIRE/RAW cells was markedly lower than in GFP/RAW cells (**Figure 5B**). These suggest that AIRE may inhibit M2c phenotype of macrophage.

AIRE expression in macrophages during polarization

Results showed the fluorescent intensity significantly after M1 induction GFP-Aire/RAW cells as compare to control group (**Figure 6**), indicat-

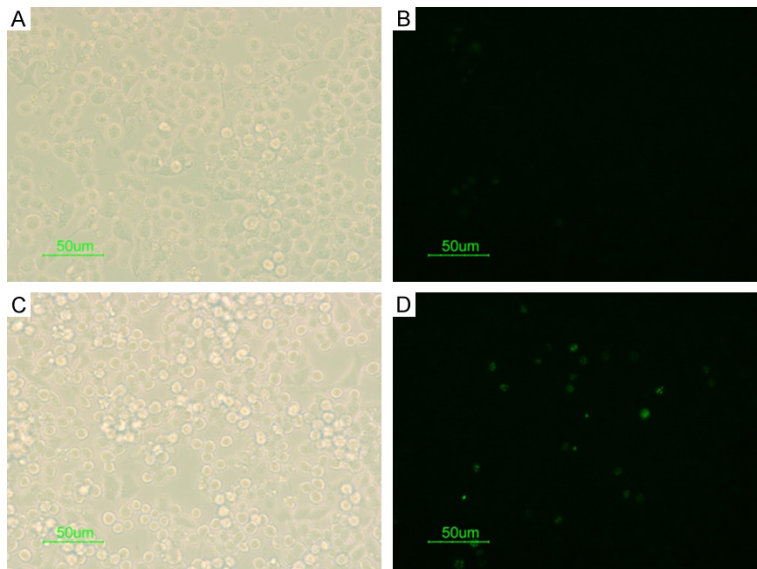


Figure 6. The fluorescence changes of GFP-AIRE/RAW cells were induced to M1. Under fluorescence microscope, the fluorescent intensity significantly increased in GFP-AIRE/RAW cells after induction of M1 phenotype when compared with control group. (A) GFP-AIRE/RAW cells under a light microscope; (B) GFP-AIRE/RAW cells under fluorescence microscope; (C) Treated GFP-AIRE/RAW cells under a light microscope; (D) Treated GFP-AIRE/RAW cells under a fluorescence microscope (A-D: 200×). Fluorescence microscope was used to detect fluorescence objectives. Light microscope was used to find the cells.

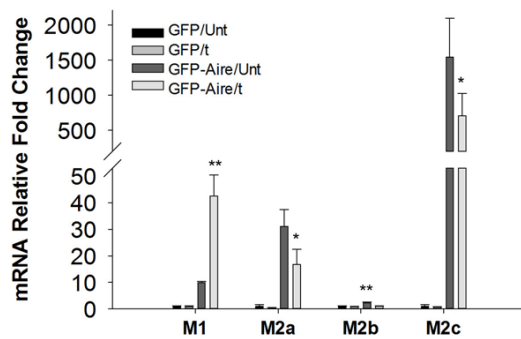


Figure 7. The expression of AIRE may affect macrophage polarization. mRNA expression of AIRE in GFP-AIRE/RAW cells and GFP/RAW cells after different treatments. AIRE mRNA expression increased significantly when GFP-AIRE/RAW cells induced to M1 compared with control group. In other subtypes of macrophage, AIRE mRNA expression in treated GFP-AIRE/RAW cells was decreased to different extents when compared with control group. Data are expressed as means \pm SD from three independent experiments. Treated GFP-AIRE/RAW cells vs untreated GFP-AIRE/RAW cells. * $P < 0.05$, ** $P < 0.01$.

ing that AIRE expression may have an impact on macrophages polarization. Therefore, AIRE mRNA expression was detected in all subtypes of macrophages by qPCR. RAW 264.7 cells

were treated as above mentioned, and then AIRE mRNA expression was detected in GFP-Aire/RAW cells and GFP/RAW cells to after M1 induction. Results are shown in **Figure 7**. The AIRE mRNA expression in treated GFP-Aire/RAW cells increased significantly when compared with control group. However, AIRE mRNA expression remained unchanged in GFP/RAW. In other subtypes of macrophage, AIRE mRNA expression in treated GFP-Aire/RAW cells was decreased to different extents when compared with control group. These data suggest that high expression of AIRE may promote the M1 differentiation of macrophages, and the low expression of AIRE may inhibit the differentiation of macrophages into M2a, M2b, and M2c phenotypes to different degrees.

Discussion

APS I, the most common non-endocrine disease is characterized by the susceptibility to Candida infection, which is difficult to explained by lack of auto-reactive T cells in the thymus. In addition, immunization of AIRE^{-/-} mice with a heterologous antigen may leads to T cell hyperproliferation [15]. These findings suggest AIRE may play a role in the peripheral tolerance. AIRE expressed in peripheral DCs may regulate T cell activity to play an important role in peripheral immune tolerance [16]. Gardner et al [17, 18] speculated that eTACs in peripheral secondary lymphoid organs could induce CD4⁺T cell apoptosis and CD8⁺T cell inactivation, affecting the peripheral immune tolerance. These suggest the clinical manifestations of APS I are closely related to the change in peripheral immune tolerance. Our previous study has shown that AIRE in the macrophage-pathogen recognition plays an important role in the peripheral immune tolerance [5].

Macrophages, under different inflammatory conditions can be polarized to different subtypes, and acquire corresponding functions.

Macrophages of different phenotypes may secrete specific cytokines, which has been studied extensively. When pathogenic microorganisms invade the body, monocytes will be recruited to the lesion site via peripheral circulation, and PAMPs such as LPS can be recognized by TLRs, promoting polarization of macrophages into M1 phenotype (classically activated macrophages, CAM), as earlier infection of the immune response to infection. M1 macrophages can secrete a large amount of inflammatory mediators, such as TNF- α and IL-1 and NO, which may exert anti-bacteria and anti-tumor effects [6]. Our results suggest that when RAW 264.7 cells were polarized to M1 phenotype, the mRNA expression of these mediators in treated GFP-Aire/RAW cells significantly increased as compared to GFP/RAW cells. These indicate that AIRE may promote differentiation of RAW 264.7 cells to into M1, which enhance their capability to kill pathogens, participating in the adaptive immune response.

While macrophages can be polarized to M1. Th2 cytokines can induce M2a phenotype of macrophages, which is involved in the regulation of wound healing and fibrosis and plays an important role in the immune regulation by inhibiting over-reaction to inflammation [7]. M2a macrophages are characterized by the expression of Arg1. Our study showed the treated GFP-Aire/RAW had significantly reduced Arg1 mRNA expression as compared to treated GFP/RAW cells. Moreover, the urea concentration was measured to assess Arg1 activity in both cell types. Results showed the change in Arg1 protein expression was similar to that in its mRNA. These data findings indicate that AIRE may play an important role in macrophages to polarization into M2a phenotype. Arg1 inhibited NO synthesis, regulated arginine metabolism and fibrosis, and restricted T cell proliferation [19]. M2a macrophages can not only promote wound healing, but also may have an important role in the development of inflammation. Therefore, AIRE may influence macrophage polarization into M2a that macrophage polarization into Arg1 expression, to mitigate antagonistic effect on M1, and regulate the immune response to inflammation.

Immune complexes and TLRs agonist can induce macrophages polarization into M2b phenotype that inhibits inflammation and regu-

late the immune response [13, 20]. M2b is a special subtype of M2. M2b macrophages may secrete a large amount of IL-10, and a small amount of IL-12. Though M2b macrophages can secrete a large amount of inflammatory factors, they also can protect against LPS toxicity [8]. Our findings suggested IL-10 mRNA expression in GFP-Aire/RAW cells significantly decreased as compared to GFP/RAW cells. The expression of IL-10 in the supernatant was measured by ELISA, and the change was consistent with its mRNA expression. IL-10 as an anti-inflammatory factor can regulate the immune response to inflammation. IL-10 deficiency may affect the immune function of DCs [21]. Our results suggested AIRE could inhibit differentiation of RAW 264.7 cells into M2b phenotype, down-regulate IL-10 expression, and play an important role in adaptive immune response.

M2c is a discrete subtype of M2, and can be induced by glucocorticoids and IL-10. M2c macrophages can swallow up early apoptosis of neutrophil and release of anti-inflammatory cytokines such as IL-10, plays an important role in innate immunity [9, 14, 22]. Our results indicated that IL-10 mRNA expression in M2c macrophages was similar to that in M2b macrophages. In order to avoid the interference by residual IL-10 in the cell supernatant, Gas6 secreted by M2c macrophages was detected. Results indicated that Gas6 expression in treated GFP-Aire/RAW cells significantly decreased when compared with GFP/RAW cells. Gas6 may influence IL-10 expression in the M2c macrophages via though GAS6/MerTK signaling pathway. Therefore, AIRE may inhibit the differentiation of RAW 264.7 cells into M2c phenotype, down-regulate IL-10 expression, and play a crucial role in immune response.

In the M1 polarization, the fluorescent intensity significantly increased in GFP-Aire/RAW cells after induction of M1 phenotype when compared with control group. Therefore, AIRE expression may have an impact on the polarization of macrophages. NF- κ B plays a crucial role in the establishment of immune tolerance, including both central tolerance and the peripheral function of regulatory T (Treg) cells, and defect or deregulated activation of NF- κ B may contribute to autoimmunity and inflammation [23]. NF- κ B is a key molecule involved in inflammation and may influence the polarization of

macrophages. P50 NF- κ B plays a divergent transcriptional role in the transcription of polarization process, by promoting Pol II recruitment on M2 promoter genes (e.g. CCL17 and Arginase I) and limiting its recruitment by M1 promoter genes ((iNOS, TNF- α) [24]. Meanwhile, Co-activator (CBP) also affects the binding of NF- κ B to the promoter of NF- κ B target gene [25]. Interaction between AIRE and CBP may influence the expression of related genes expressed in mTEC [26]. Therefore, AIRE may synergize with CBP to affect macrophage polarization through regulating the expression of NF- κ B related target genes expression in inflammation. In order to confirm this hypothesis, AIRE mRNA expression was detected in different macrophage subtypes. Our results showed AIRE mRNA expression in treated GFP-Aire/RAW cells increased significantly when compared with control group. However, AIRE mRNA expression remained unchanged in GFP/RAW cells. In other subtypes of macrophages, AIRE mRNA expression in treated GFP-Aire/RAW cells decreased to different degrees when decreased compared with control group. We speculate that AIRE expression may affect the macrophage polarization, which play a key role in maintaining peripheral immune tolerance and may enhance the killing of pathogenic microorganisms. Whether does AIRE synergize with other molecules to affect the immune response is needed to be confirmed in future studies.

Conclusion

Our study suggests that AIRE may promote the differentiation of RAW 264.7 cells into M1 phenotype, antagonize for M2 polarization, and enhance the killing of pathogenic microorganisms, playing an important role in the inflammatory immune response. This may partially explain the susceptibility of APECED patients to Candida infection. Meanwhile, AIRE expression has an impact on the polarization of RAW 264.7 cells, but more studies are warranted. In addition, the function of AIRE is needed to be in hematopoietic stem cells *in vivo*.

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Disclosure of conflict of interest

None.

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