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Aire deficient dendritic cells promote the T follicular helper cells differentiation

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ABSTRACT

Autoimmune regulator (Aire), primarily expressed in medullary thymic epithelial cells (mTECs), maintains central immune tolerance through the clearance of self-reactive T cells. Aire can also be expressed in dendritic cells (DCs), and DCs can mediate T follicular helper (T_{FH}) cell differentiation and self-reactive B cell activation through inducible costimulator molecule ligand (ICOSL) and interleukin 6 (IL-6), which can cause autoimmune diseases. To confirm whether Aire in DCs affects T_{FH} cell differentiation and to determine the role of Aire in the maintenance of peripheral immune tolerance, this study observed the effects of Aire deficiency on T_{FH} cells using Aire knockout mice. The results showed that Aire deficiency caused increased number of T_{FH} cells, both *in vivo* and *in vitro*. Further studies showed that Aire deficiency promoted T_{FH} differentiation through the upregulation of ICOSL and IL-6 in DCs. Thus Aire could suppress the expression of ICOSL and IL-6 to inhibit T_{FH} cell differentiation.

1. Introduction

Autoimmune regulator (Aire) is a transcription factor, and a single gene mutation in Aire results in autoimmune polyglandular syndrome type 1 (APS1), which shares major characteristics with many autoimmune diseases, such as the involvement of endocrine glands and chronic mucosal candida infection (Betterle et al., 1998), indicating that Aire plays important roles in the maintenance of autoimmune tolerance and preventing the development of autoimmune diseases. It is well known that autoimmune diseases are caused by the combined effects of self-reactive T cells and self-reactive B cells. Multiple studies have already confirmed that Aire is primarily expressed in medullary thymic epithelial cells (mTECs), and it prevents the development of autoimmune diseases through the clearance of self-reactive T cells (Akirav et al., 2011). In addition, Aire can also be expressed in dendritic cells (DCs) (Gardner et al., 2013), our previous studies showed that the transplantation of the Aire overexpressing mouse DC cell line DC2.4 educated T lymphocytes into streptozotocin-induced type 1 diabetes mellitus model mice could delay disease progression, suggesting that Aire expressed in peripheral DCs might be involved in the maintenance of peripheral immune tolerance (Li et al., 2015). However, the specific mechanism underlying this effect and whether it is caused by inhibiting the activation of self-reactive B lymphocytes are still not clear.

It has been shown that T follicular helper (T_{FH}) cells are an important subpopulation that facilitates the activation of self-reactive B cells. An abnormal level of T_{FH} cells has been closely associated with the development of autoimmune diseases (Blanco et al., 2016; Meguro et al., 2015; Li et al., 2013; Kim et al., 2017). T_{FH} cells are localized in lymphoid follicles, and their characteristic transcription factor is B cell lymphoma 6 (Bcl-6), which is necessary for T_{FH} differentiation. In contrast, B lymphocyte-induced maturation protein 1 (Blimp1), an antagonist of Bcl-6, inhibits T_{FH} differentiation, therefore, these cells express low or no Blimp1 (Yu et al., 2009). T_{FH} cells primarily exert their influence through the secretion of interleukin 21 (IL-21), and they express high levels of various biomarker molecules, including C-X-C chemokine receptor type 5 (CXCR5), inducible costimulatory molecule (ICOS), and programmed death 1 (PD-1), which interact with corresponding ligands or receptors on the surface of B cells, facilitating the production of high-affinity antibodies in B cells (Crotty, 2014).

T_{FH} cell differentiation is a multistage, multifactorial process, and it starts at initial DCs priming of a naïve $CD4^+$ T cell. In mouse models, the initial stage of T_{FH} cell differentiation is regulated by interleukin 6 (IL-6), ICOS and T cell receptor (TCR) signals strength. IL-6, secreted by DCs, is the earliest non-TCR signal, it interacts with its corresponding receptor to induce Bcl-6 expression, which is a key transcription factor involved in the induction of T_{FH} differentiation (Nurieva et al., 2009).

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IL-6 deficiency in DCs results in poor T_{FH} cell differentiation (Chakarov and Fazilleau, 2014; Choi et al., 2013). Inducible costimulator molecule ligand (ICOSL) is primarily expressed in DCs, B cells, and other antigen-presenting cells (APCs), and it belongs to the B7 family. ICOS, the ligand for ICOSL, is only expressed in activated T cells and belongs to the CD28 family. The ICOSL-ICOS interaction can regulate humoral immunity and germinal center reaction. This signaling pathway is very important for mediating the production of T_{FH} cells in mice. Blocking ICOSL in DCs can reduce the expression levels of CXCR5 and ICOS in $CD4^+$ T cells (Shin et al., 2015). T_{FH} cell development has been reported to be inhibited in ICOS deficient mice and humans (Akiba et al., 2005; Bossaller et al., 2006).

It has been shown that spleen DCs derived from Aire deficient mice are able to more strongly induce T_{FH} cell differentiation than those from wild-type mice (Lindmark et al., 2013). Our previous studies found that T_{FH} cells were significantly increased in the spleens of Aire knockout mice, and ICOSL expression levels in bone marrow derived DCs were also significantly increased (data not shown). However, whether Aire influences T_{FH} cell differentiation through the regulation of ICOSL expression in DCs is still not clear.

Based on the above background, we performed the following studies. 1. We observed the number of T_{FH} and germinal center B cells, as well as Bcl-6 expression in $CD4^+$ T cells in the lymph nodes and spleen, the expression of ICOSL and IL-6 in DCs in Aire knockout mice infected with the FM1 strain of the H1N1 influenza virus. We try to investigate the relationships between Aire expression and both ICOSL expression and IL-6 secretion in DCs and changes in the number of T_{FH} cells. 2. We observed the effects of bone marrow DCs (BMDCs) from Aire knockout mice ($Aire^{-/-}$-BMDCs) on T_{FH} cell differentiation using co-culture technology *in vitro*, and we examined the expression levels of ICOSL and IL-6 in $Aire^{-/-}$-BMDCs to study the role of Aire in T_{FH} cell differentiation and the possible associated mechanisms.

2. Materials and methods

2.1. Experimental animals

B6.129S2-*Aire*$^{tm1.1Doi}$/J mice aged 4–6 w, were purchased from Nanjing Biomedical Research Institute of Nanjing University, China (SPF grade). C57BL/6 J mice aged 4–6 w, were purchased from Experiment Animal Centre of Jilin University, China (SPF grade). All animal experiments were performed with the permission of the Experimental Animal Ethics Committee of Jilin University.

2.2. Establishment of the H1N1 influenza virus infected mouse model

The FM1 strain of the H1N1 influenza virus was a gift from the Laboratory of Molecular Biology, College of Basic Medicine Sciences, Jilin University. The virus titre was $10^{4.25}$ TCID50·ml^{-1}. Mice were deeply anesthetized through the intraperitoneal injection of 10% chloral hydrate at 4 μl g^{-1}. The virus was diluted by 10^6-fold and applied by the intranasal infection of 50 μl to each mouse. Mice were injected with normal saline as control group. Mice were housed normally in independent isolation cages.

2.3. Collection of peripheral blood, spleens, and lymph nodes of virus-infected mice

After 8 days of H1N1 virus infection, the blood were obtained from mice and collected in Eppendorf (EP) tubes Next, the spleens, skin-draining lymph nodes (SLNs), and mesenteric lymph nodes (MLNs) were collected for grinding, erythrocytes lysis, filtration, centrifugation for detection.

2.4. Generation of mouse BMDCs

Bone marrow cells from mice were cultured in 10% 1640 culture medium (Gibco, USA), containing the cytokines GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) (PeproTech, USA). The culture medium was changed every other day. BMDCs were collected on day 6. Cells were then stimulated for 24 h using 1 μg/ml lipopolysaccharide (LPS) and 100 μg/ml olyinosinic-polycytidylic acid (poly(I:C)).

2.5. RNA isolation and RT-qPCR

Total RNA was isolated from the BMDCs using RNAiso™ PLUS (TaKaRa, Japan). According to the amount of the precipitate, an appropriate amount of diethyl pyrocarbonate (DEPC) treated distilled water was added. The concentration of RNA was measured using an ultraviolet spectrophotometer (Biotek, USA). RNA, at 1 μg, was used as the template, and reverse transcription was performed. The RT-qPCR reaction conditions were 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The following primer sequences were used: GAPDH:F: 5′-GACTTCAACAGCAACTCCCACTC-3′,R:5′-TAGCCGTATTCATTGTCATACCAG-3′;IL-6:F:5′-GAAACCGCTATGAAGTTCCTCTCTG-3′,R:5′-GTATCCTCTGTGAAGTCTCCTCTCC-3′;andICOSL:F:5′-AGCTCCATGTTTCTAGCGGGTTC-3′,R:5′-ACCATTGCACCGACTTCAGTCTC-3′.The ABI 7300 (Applied Biosystems, USA) was used for detection to obtain Ct values. Data analysis was performed using the $2^{-\triangle\triangle Ct}$ algorithm.

2.6. Flow cytometry

Cells were collected into 1.5 ml EP tubes, and washed with phosphate-buffered saline (PBS) twice. 100 μl PBS was added to resuspend cells. An appropriate amounts of FITC-anti-PD1, PE-anti-CXCR5, PE-Cy7-anti-CD4, FITC-anti-GL7, PE-Cy7-anti-CD45R, PE-anti-Fas, PE-anti-CD11b, PE-Cy7-anti-CD11c, or Biotin-anti-CD275 (eBioscience, San Diego, CA, USA) was added, followed by incubation on ice for 20 min in the dark. Cells were washed with PBS and resuspended. The APC-Streptavidin antibody was added and incubated on ice for 20 min. In the meantime, the cells were fixed with fixation/permeabilization concentrate and diluent (eBioscience) for 45 min. Subsequently, the cells were treated with 0.1% saponin(Sigma-Aldrich, St. Louis, MO, USA), APC-anti-IL-6, APC-anti-Bcl-6 (eBioscience) on ice for 50 min. Cells were washed with PBS once and resuspended in 200 μl 2% paraformaldehyde. Detection was performed in a BD FACS Calibur flow cytometer.

2.7. Co-culture of the BMDCs with $CD4^+$ T cells

Splenocytes of C57BL/6 J mice were collected. Cell sorting was performed according to the instructions of the naïve $CD4^+$T cell sorting reagent kit (Stemcell, Canada). BMDCs (3×10^4 per well) and naïve $CD4^+$T cells (3×10^5 per well) were added into 96-well plate, co-cultured for 72 h. Cells were harvested for flow cytometry.

2.8. ELISA

BMDCs cultured for 24 h were collected and centrifuged at 3000 rpm for the collection of supernatants. Peripheral blood samples of H1N1 immunised mice were collected and centrifuged at 3000 rpm for the collection of serum samples. An ELISA was performed according to the instructions of the ELISA sandwich reagent kit (eBioscience, San Diego, CA, USA; Jianglai Bio, China).

2.9. Haematoxylin-Eosin (HE) stain

Lung tissues were fixed in 10% formalin, dehydrated in alcohol,

cleared in xylene, embedded in paraffin, sectioned, and subject to HE staining. Sections were baked at 65℃ for 30 min and fixed. HE stained lung sections were observed under a light microscope.

2.10. Statistics

All experimental data are from at least three to six samples, and presented as the mean ± standard deviation (mean ± SD). All experiments were repeated at least three times. Data fit normal distribution, and statistical analysis was performed by the Student's *t*-test P value < 0.05 was considered statistically significant.

3. Results

3.1. The effects of Aire on T_{FH} cell differentiation in vivo

3.1.1. Establishment of a mouse model infected with the H1N1 influenza virus

To observe the effects of Aire on T_{FH} cell differentiation *in vivo*, Aire knockout mice and wild-type mice were infected with the FM1 strain of the H1N1 influenza virus. Mice were injected with normal saline as control group. To confirm whether the H1N1 infected mouse model was established successfully, peripheral blood from model mice was collected on day 8 for the detection of H1N1-IgG using ELISA. The results showed that, the serum IgG concentrations of model mice were between 4–10 μg/ml after H1N1 infection, and the concentrations in the Aire knockout mice were significantly higher than those in wild-type mice (Fig. 1A). In addition, HE staining was performed on lung tissues from model mice on day 8 after virus infection. The results showed that model mice exhibited obvious inflammatory cells infiltration, and the infiltration was more serious in the Aire knockout mice (Fig. 1B). The above results indicated that the H1N1 infected mouse model was established successfully and that the antibody response level in the Aire knockout mice was significantly higher than that in the wild-type mice.

After 8 days of H1N1 infection, A. Detection of the H1N1-IgG level in peripheral blood using ELISA. B. HE staining of mouse lung tissues. The data is representative (n = 6 for each, >6 in total) of > = 3 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.1.2. T_{FH} cell differentiation in Aire knockout mice

To further investigate the reason for the increase in anti-H1N1 antibodies observed in Aire knockout mice, the numbers of T_{FH} cells that facilitated the production of antibodies by B cells, the number of germinal center B cells and the expression of Bcl-6 in $CD4^+$ T cells in the spleens, SLNs, and MLNs from model mice (Aire knockout mice and wild-type mice) were detected on day 8 after H1N1 infection using FACS. The results showed that the numbers of T_{FH} cells in the spleens and lymph nodes from Aire knockout mice were higher than those in wild-type mice, and the differences in the lymph nodes were more significant than those in the spleens (Fig. 2A, B and C). The numbers of germinal center B cells in the spleens and lymph nodes from Aire knockout mice were also higher than those in wild-type mice (Fig. 2D, E and F), Bcl-6 in $CD4^+$ T cells in the spleens and lymph nodes from Aire knockout mice were upregulated compared with that in the wild-type mice, however, the levels of Bcl-6 in the lymph nodes from H1N1 infected mice were decreased compared with that in the uninfected mice (Supplementary figure). The decrease of Bcl-6 expression in $CD4^+$T cell in the H1N1 mice was speculated partly because of the change of microenvironment *in vivo*. Since the T subsets are phenotypic plasticity. T cell subpopulations may change with the disease progression. Bcl-6 as the key transcription factor of T_{FH} will be downregulated at the recovering process. These results suggested that Aire might inhibit the differentiation of T_{FH} and germinal center B cells. This inhibition may explain the increased antibodies in H1N1 infected Aire knockout mice.

After 8 days of H1N1 infection, the numbers of T_{FH} cells in the spleen(A), SLNs(B), MLNs(C) and the numbers of germinal center B cells in the spleen(D), SLNs(E), MLNs(F) were detected using FACS. The isotype controls were showed in Fig. 2G. The data is representative (n = 6 for each, >6 in total) of > = 3 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.1.3. ICOSL and IL-6 expression in Aire knockout mice

As the most powerful APCs, DCs can promote the activation and differentiation of naïve $CD4^+$ T cells by providing the strongest stimulus (Jenkins et al., 2001). Therefore, we speculated that Aire might influence T_{FH} cell differentiation through DCs. There are many molecules expressed in DCs that can promote T_{FH} cell differentiation, including the costimulator molecule, ICOSL and the cytokine, IL-6. ICOSL

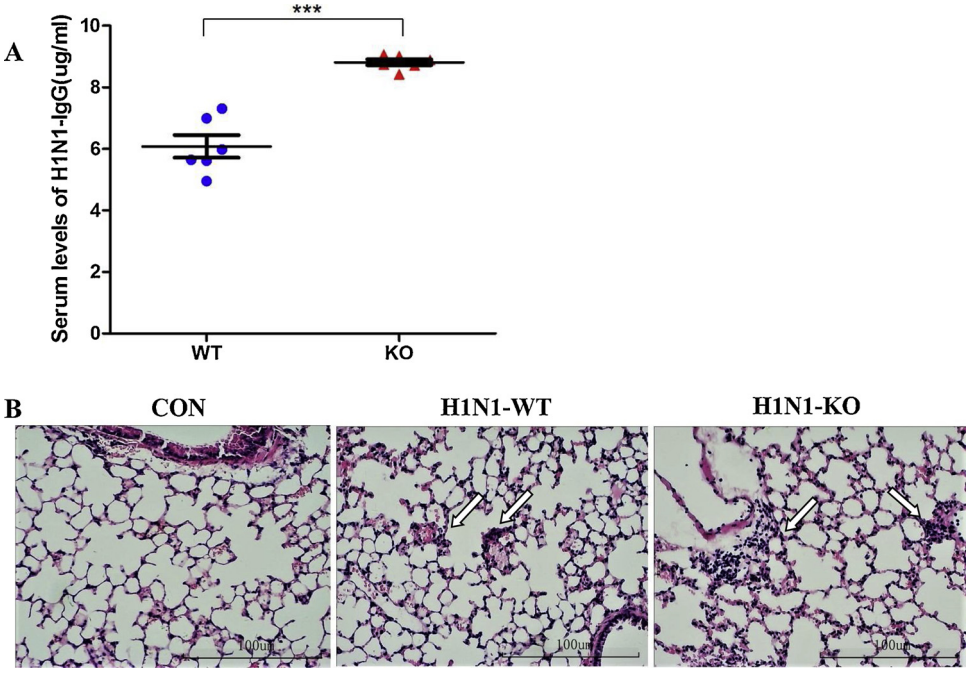

Fig. 1. Establishment of the H1N1 infected mouse model.

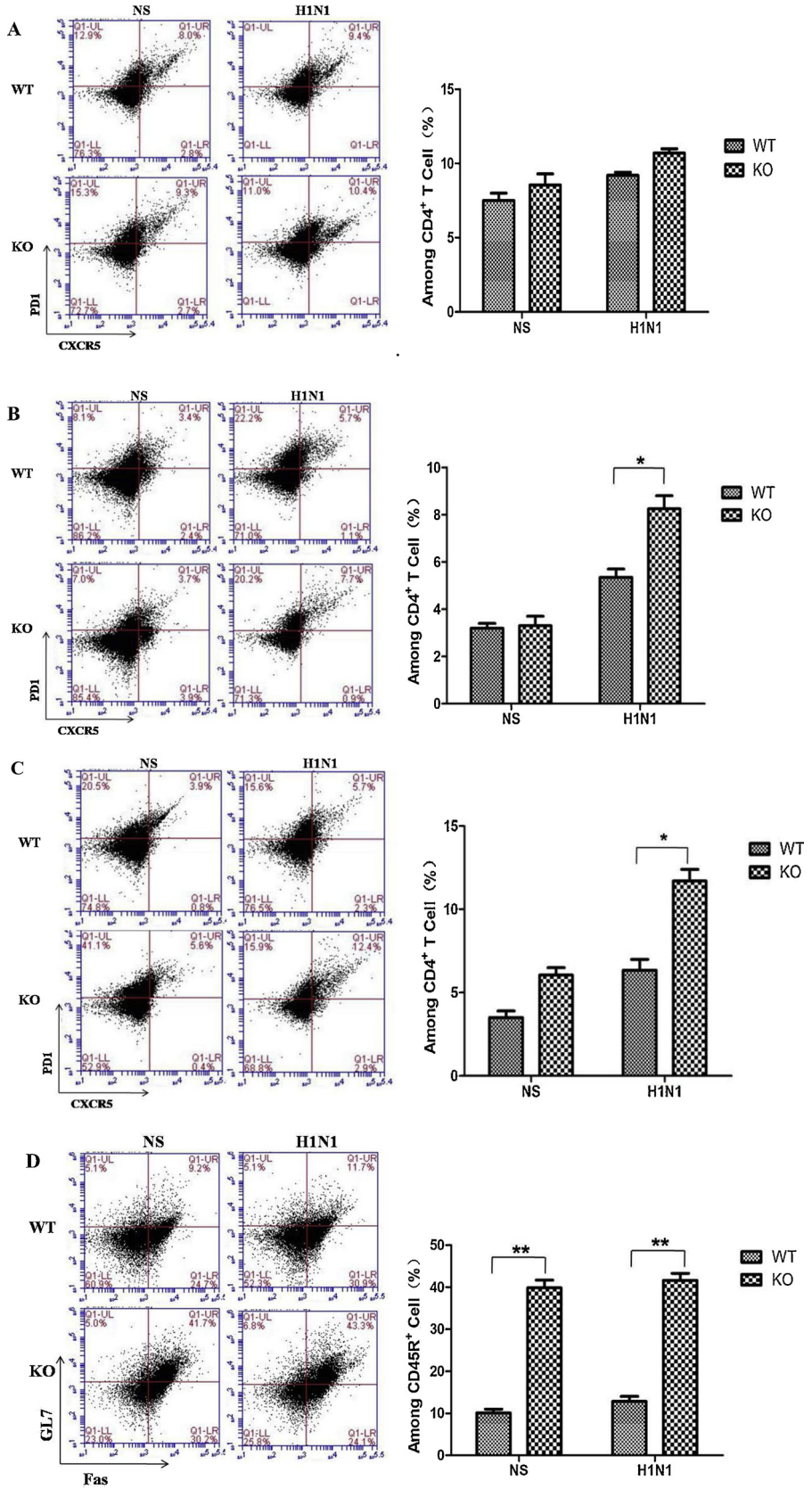

Fig. 2. The effects of Aire on T_{FH} cell differentiation.

expression levels in DCs from the spleens, SLNs, and MLNs were detected on day 8 after H1N1 infection using FACS, and IL-6 expression levels in DCs from the spleens after LPS stimulation for 6 h were detected using FACS. The data showed that ICOSL in DCs from the spleens and lymph nodes of knockout mice were significantly upregulated compared with that in wild-type mice (Fig. 3A, B and C), and the

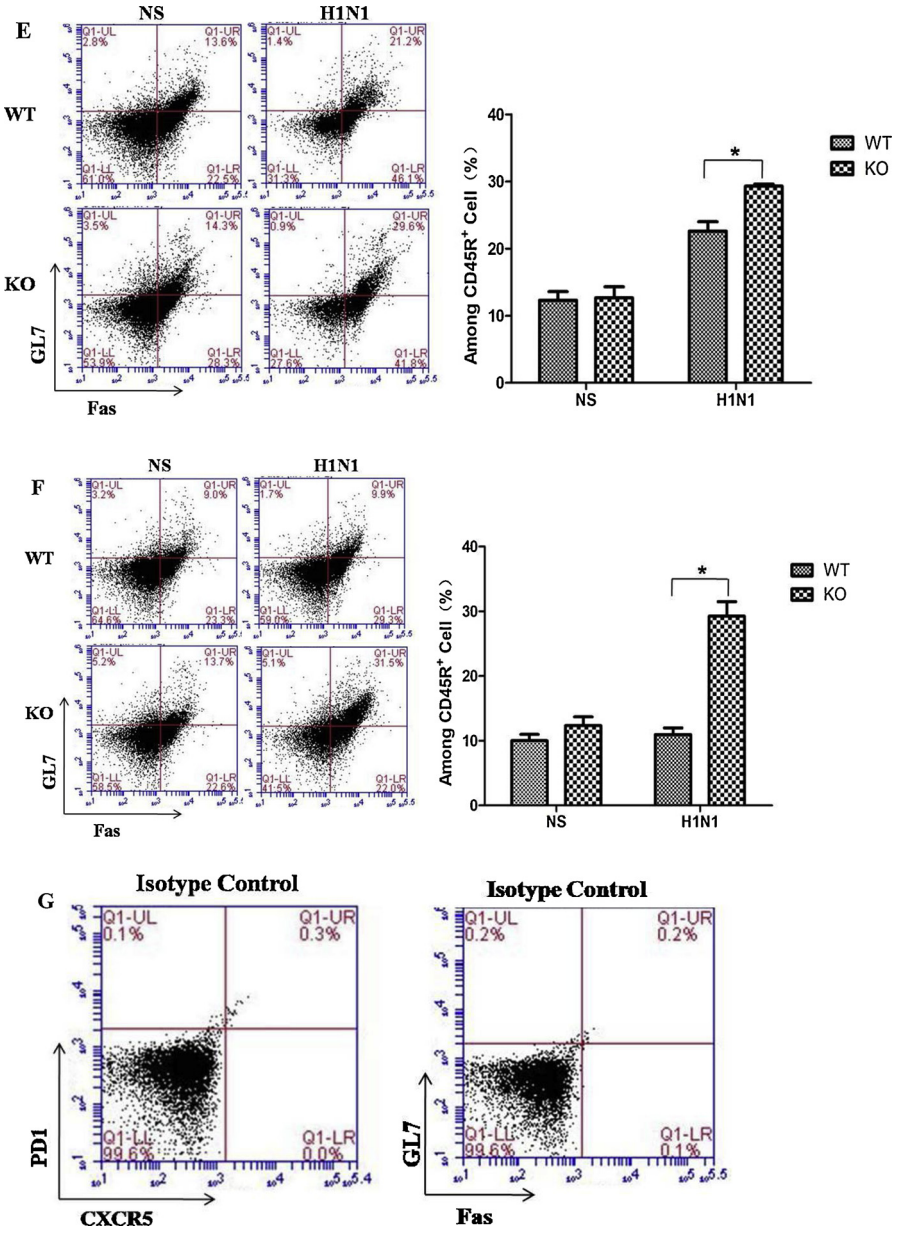

Fig. 2. (*continued*)

levels of IL-6 in DCs from the spleens of knockout mice were significantly increased than those in wild-type mice (Fig. 3D). These results suggested that Aire might inhibit ICOSL and IL-6 expression in DCs *in vivo*.

After 8 days of H1N1 infection, the expression levels of ICOSL in DCs from the spleens(A), SLNs(B), and MLNs(C) were detected using FACS; the expression levels of IL-6 in DCs from the spleens(D) were detected using FACS. The isotype controls were showed in Fig. 3E. The data is representative (n = 6 for each, >6 in total) of > = 3 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.2. The effects of Aire$^{-/-}$-BMDCs on T_{FH} cell differentiation in vitro

The above results showed that Aire was related to ICOSL expression in DCs and IL-6 production and to the numbers of T_{FH} cells in Aire knockout mice. However, it was not clear whether a direct association existed. Therefore, we further observed the effects of Aire$^{-/-}$-BMDCs on T_{FH} cell differentiation *in vitro*, and examined the expression levels of ICOSL and IL-6 in BMDCs derived from Aire knockout mice to investigate the effects of Aire on T_{FH} cell differentiation and the possible underlying mechanisms.

3.2.1. Aire$^{-/-}$-BMDCs influenced the differentiation of T_{FH} cells

To observe the effects of Aire$^{-/-}$-BMDCs on T_{FH} cell differentiation both at a resting and stimulated state, two types of stimuli were selected: (1) LPS, one of the major cell wall components of Gram-negative bacteria that present important antigens to DCs after infection; and (2) poly(I:C), a double-stranded RNA analogue and a virus infection associated molecular pattern. Poly(I:C) can be recognised by toll-like receptor 3 (TLR3) to induce nuclear factor kappa B (NF-kB) activation and cytokine production. We performed naïve CD4$^+$T cell sorting using magnetic beads. The purity of cells detected using FACS could reach 98% (data not shown). Resting BMDCs, stimulated BMDCs with 1 μg/ml LPS for 24 h or 100 μg/ml poly(I:C) for 24 h were co-cultured with naïve CD4$^+$ T cells for 72 h. The results showed that Aire$^{-/-}$-BMDCs induced the differentiation of T_{FH} cells (Fig. 4A and B), suggesting that Aire might inhibit T_{FH} cell differentiation by BMDCs.

Stimulated Aire$^{-/-}$-BMDCs with either 1 μg/ml LPS (A) or 100 μg/

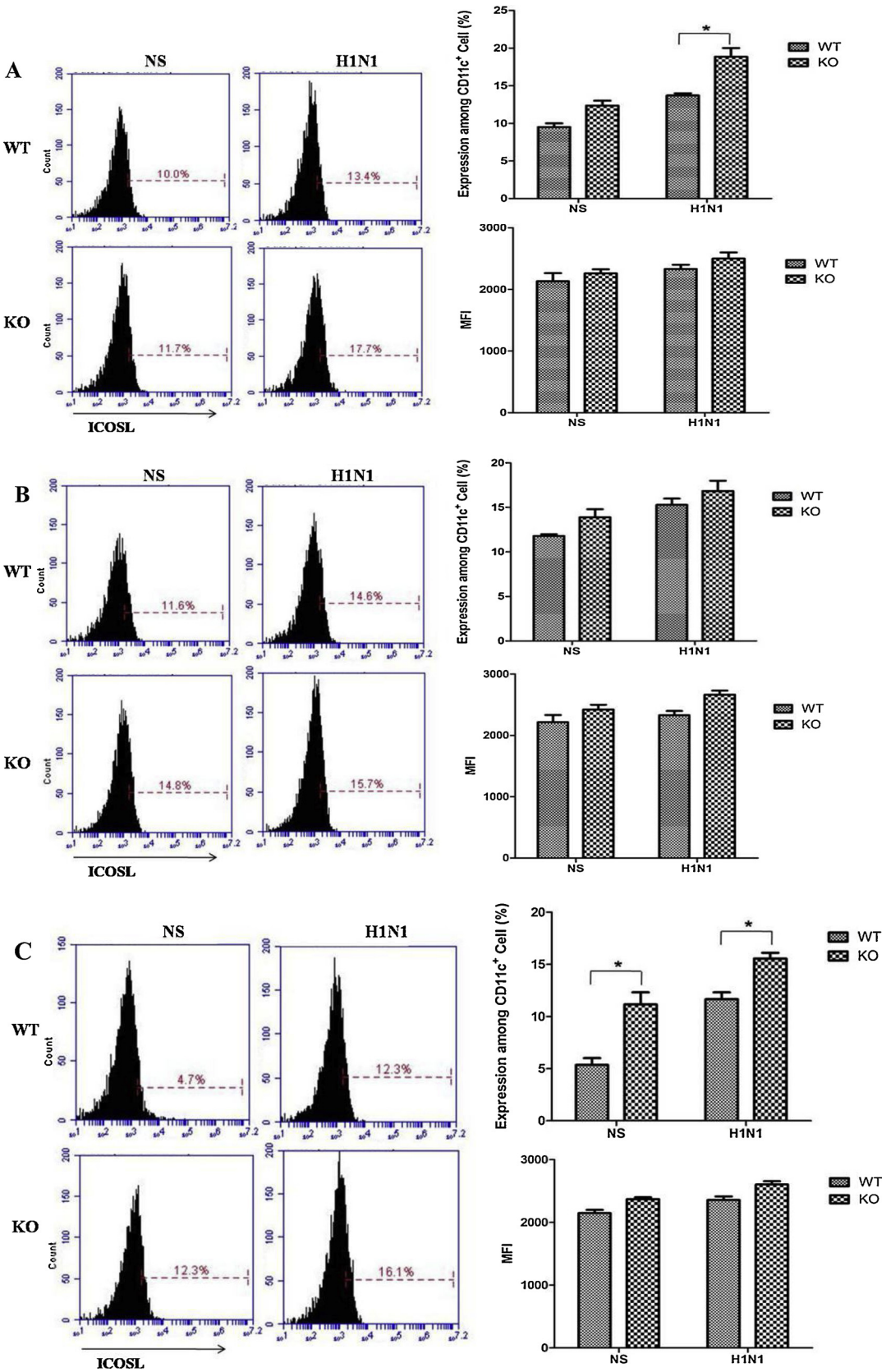

Fig. 3. The effects of Aire on the expression levels of ICOSL and IL-6.

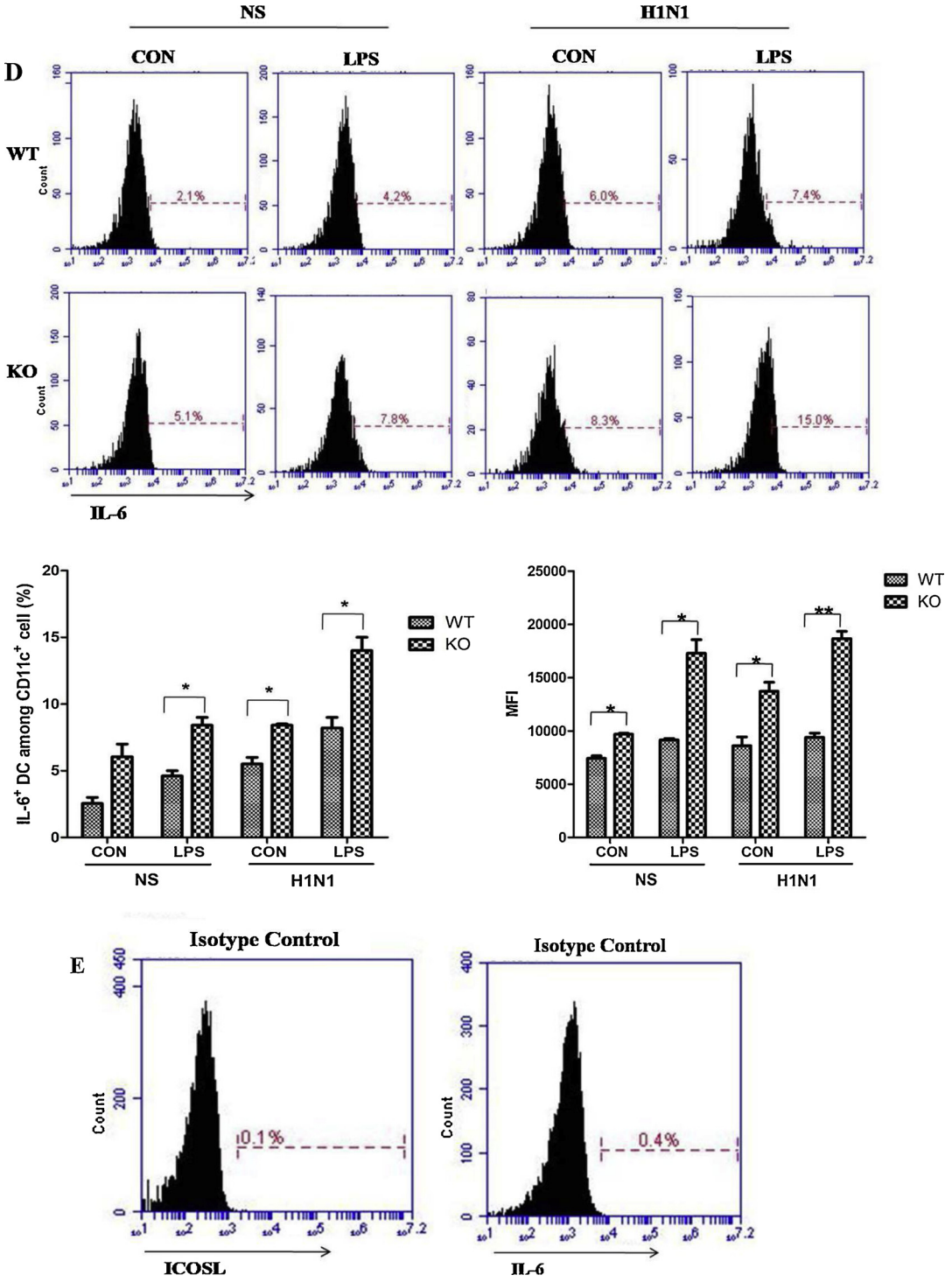

Fig. 3. (*continued*)

ml poly(I:C) (B) for 24 h were co-cultured with naïve $CD4^+$T cells for 72 h. The conditions of T_{FH} cell differentiation were detected using FACS. The isotype control was showed in Fig. 4C. The data from a minimum of three experiments were presented as the mean values. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.2.2. Expression of ICOSL and IL-6 in Aire$^{-/-}$-BMDCs

DCs could promote T_{FH} cell differentiation through ICOSL and IL-6. Our study showed that the numbers of T_{FH} cells in the spleens and lymph nodes of Aire knockout mice and the expression levels of ICOSL in DCs and IL-6 levels in serum all increased compared to those of the control group *in vivo*. The above results showed that Aire$^{-/-}$-BMDCs promoted T_{FH} cell differentiation *in vitro*. Therefore, we speculated that Aire might influence T_{FH} cell differentiation through the two above mentioned molecules, ICOSL and IL-6. We observed the effect of Aire on the expression of ICOSL and IL-6 in BMDCs using RT-qPCR, FACS, and ELISA. The RT-qPCR results showed that the ICOSL expression level in Aire$^{-/-}$-BMDCs significantly increased after LPS stimulation for 6 h compared to that in the control group (Fig. 5A). In addition, after poly (I:C) stimulation for 24 h, IL-6 expression in Aire$^{-/-}$-BMDCs also significantly increased compared to that in the control (Fig. 5B). At the protein level, the detection of ICOSL in DCs using FACS and the

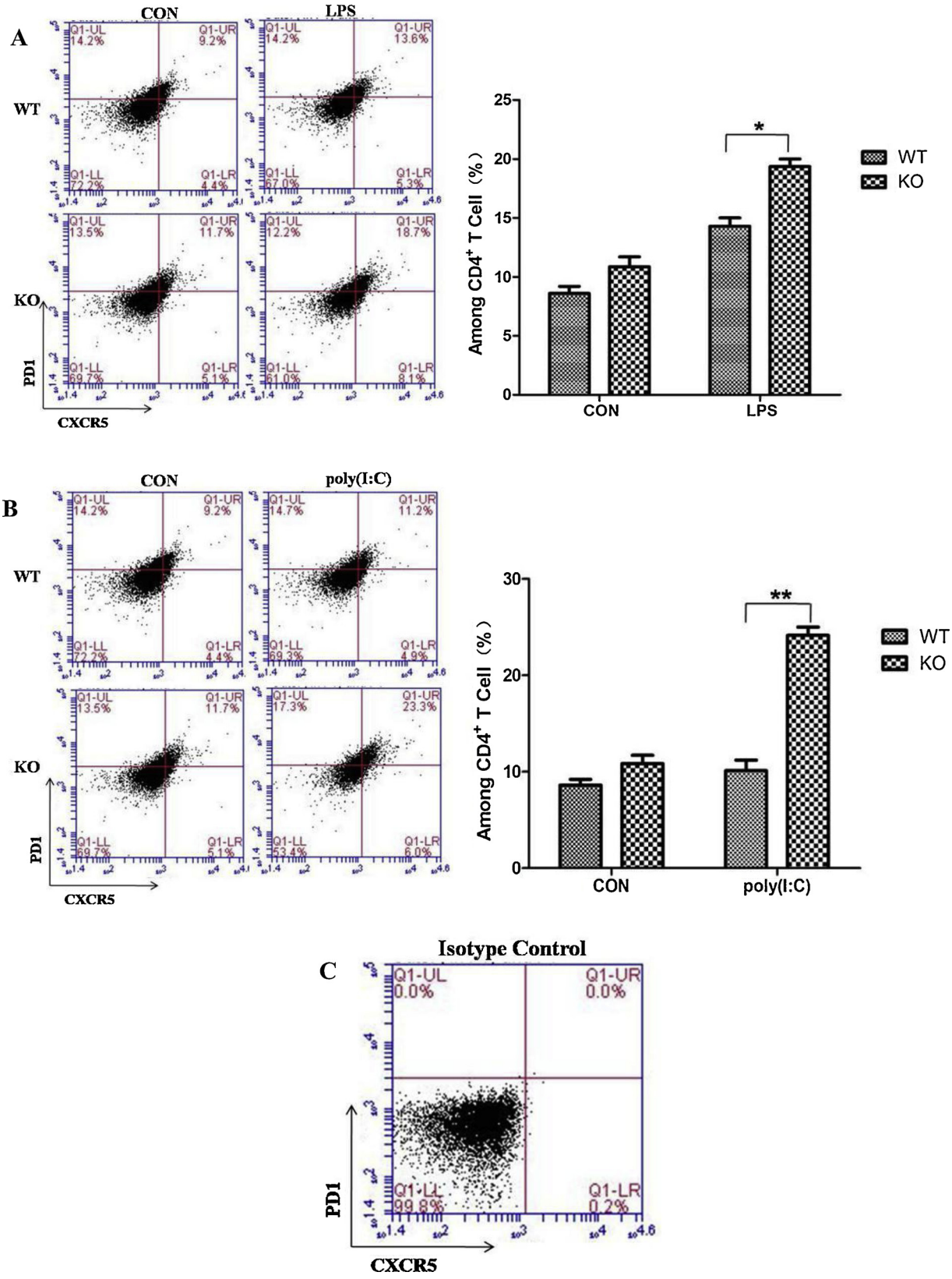

Fig. 4. The effects of Aire$^{-/-}$-BMDCs on T_{FH} cell differentiation.

detection of IL-6 in the cell supernatant using ELISA also showed the same trend (Fig. 5C and D). These results indicated that Aire might inhibit ICOSL and IL-6 expression in BMDCs.

A. Detection of the expression levels of ICOSL in BMDCs stimulated with 1 μg/ml LPS for 6 h using RT-qPCR. B. Detection of the expression levels of IL-6 in BMDCs stimulated with 100 μg/ml poly(I:C) for 24 h using RT-qPCR. C. Detection of the expression levels of ICOSL in BMDCs stimulated with 1 μg/ml LPS for 24 h using FACS. D. Detection of the

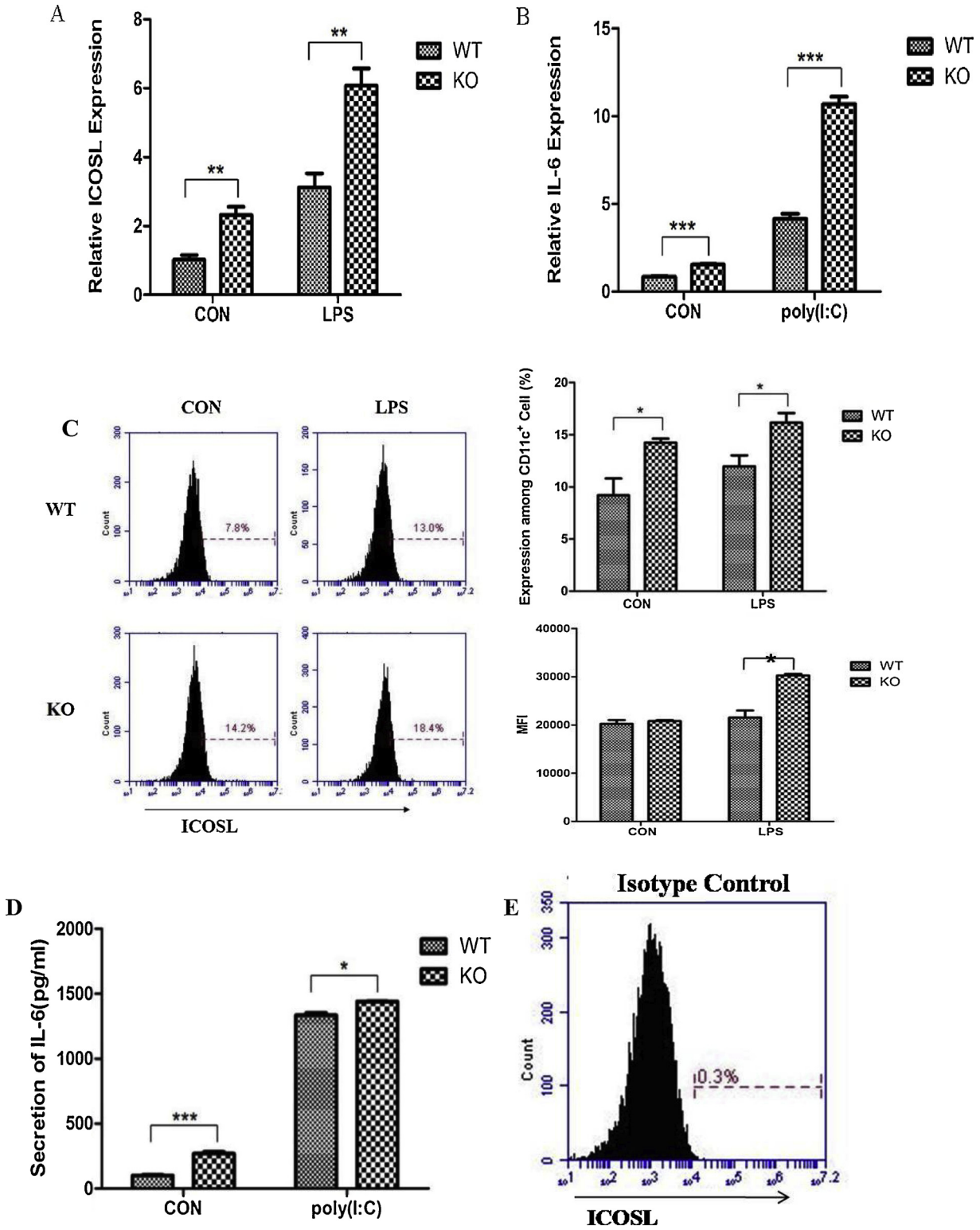

Fig. 5. The effects of Aire on the expression levels of ICOSL and IL-6 in BMDCs.

expression levels of IL-6 in BMDCs stimulated with 100 μg/ml poly(I:C) for 24 h using ELISA. The isotype control was showed in Fig. 5E. The data from a minimum of three experiments were presented as the mean values. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.2.3. The effects of Aire on T_{FH} cell differentiation through the regulation of ICOSL and IL-6 expression in BMDCs

To further confirm that Aire knockout DCs promoted T_{FH} cell differentiation through ICOSL and IL-6, supernatants from Aire$^{-/-}$-BMDCs were neutralized using ICOSL or IL-6 antibodies, and then Aire$^{-/-}$-BMDCs were co-cultured with naïve CD4^{+}T cells to observe the differentiation of T_{FH} cells. The results showed that, after blocking with ICOSL or IL-6 antibodies, the numbers of T_{FH} cells in the Aire knockout and control groups were both reduced, and there was no difference between these two groups (Fig. 6A and B). These results indicated that Aire inhibited T_{FH} cell differentiation through the

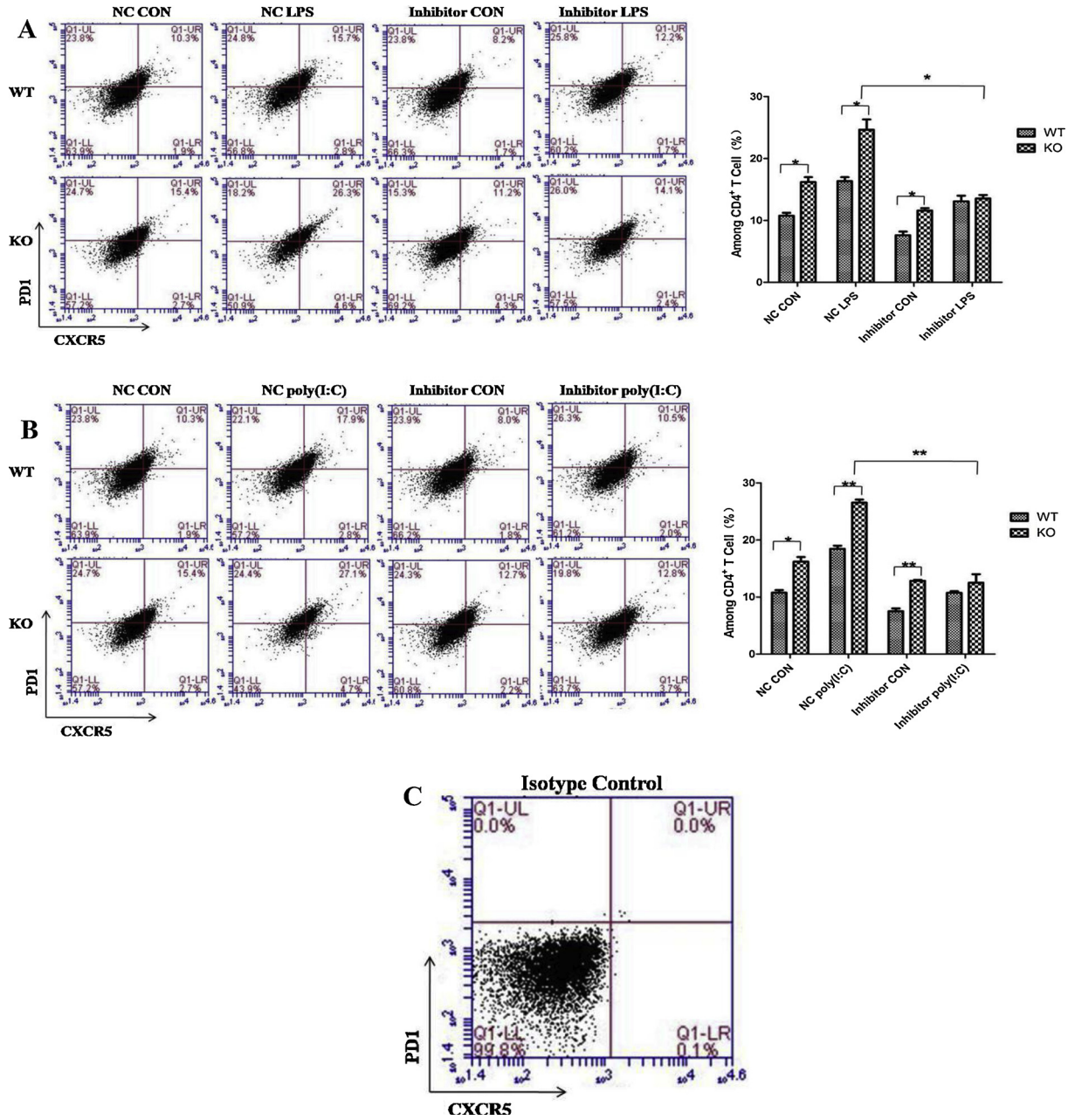

Fig. 6. The effects of Aire on T_{FH} cell differentiation through ICOSL and IL-6 in BMDCs.

downregulation of ICOSL and IL-6 expression in BMDCs.

The supernatant from BMDCs was antagonised by 20 μg/ml ICOSL antibody (A) and 50 μg/ml IL-6 antibody(B), and then the cells were co-cultured with $CD4^+$T cells. The conditions of T_{FH} cell differentiation were detected using FACS. The isotype control was showed in Fig. 6C.The data from a minimum of three experiments were presented as the mean values. *P < 0.05, **P < 0.01, and ***P < 0.001.

4. Discussion

Aire was first discovered in APS1 patients as a transcription factor 20 years ago. Aire is involved in the deletion of self-reactive T cells through the regulation of peripheral tissue restricted antigen (TRA) expression in mTECs and can induce the production of regulatory T cells to maintain central immune tolerance (Yang et al., 2015). However, the function and specific mechanisms of Aire in peripheral immune tolerance remain limited.

As immune cells that play central regulatory roles in immune responses in the body, DCs are currently known as the group of APCs that have the most powerful functions. Different subpopulations and different development stage of DCs have different phenotypic characteristics, secrete different cytokines, and play different roles in the immune system. DCs can secrete cytokines and express co-stimulator molecules to influence T cell differentiation (Grakoui et al., 1999; Langenkamp et al., 2000).

In 2000, (Breitfeld et al. (2000)) and (Schaerli et al. (2000)) discovered a $CD4^+$T cell subpopulation localized in the human tonsil germinal center. This population expressed high levels of CXCR5, could migrate to peripheral lymphoid follicles to help B cells, and played critical roles in the formation of the germinal center, the maturation of antibody affinity, the production of high-affinity antibodies, and the generation of memory B cells. This subpopulation was called T follicular helper (T_{FH}) cells. T_{FH} cells can help maintain normal immune responses and homeostasis in the body, while abnormalities in the

number and function of T_{FH} cells can result in immune disorders and induce various autoimmune diseases, such as type 1 diabetes mellitus (Ferreira et al., 2015), systemic lupus erythaematosus (Choi et al., 2015; Zhang et al., 2015), rheumatoid arthritis (Wang et al., 2013; Arroyo-Villa et al., 2014), Sjogren's syndrome (Szabo et al., 2016), and myasthenia gravis (Luo et al., 2013).

Our study *in vivo* showed that the percentage of T_{FH} cells and germinal center B cells in the peripheral lymphoid tissues of Aire knockout mice was higher than that of wild-type mice. The study *in vitro* showed that Aire$^{-/-}$-BMDCs could induce the differentiation of T_{FH} cells, indicating that Aire played a role in immune tolerance through the inhibition of T_{FH} differentiation by DCs. The study reported by Lindmark et al. showed that, compared to wild-type mice, the loss of Aire in 33D1^{+}DCs led to reduced levels of the chemokine CXCL12, causing the retention of excessively activated B cells in splenic follicles. In addition, the ICOSL expression levels on Aire$^{-/-}$-33D1^{+} DCs increased to eventually promote T_{FH} cell proliferation and the recruitment of germinal center B cells (Lindmark et al., 2013). The study reported by Zhao showed that, compared to the control group, myasthenia gravis patients had reduced Aire expression in peripheral blood and a significant increase of the number of T_{FH} cells which was positively correlated with the disease severity (Zhao et al., 2018). These results were consistent with our study results. Our study showed that Bcl-6 in CD4^{+} T cells in the spleens and lymph nodes from Aire knockout mice were upregulated compared with that in the wild-type mice, which was consistent with the changes in the T_{FH}, however, the levels of Bcl-6 in the lymph nodes from H1N1 infected mouse model were decreased compared with that in the control group, the possible reason is that after H1N1 infection, T_{FH} rises and feedback inhibition occurs over time, resulting in a decrease in BCL-6 levels.

The above results prompted us to further investigate the mechanisms underlying the effects of Aire on T_{FH} cell differentiation. The results showed that ICOSL expression in DCs in the spleen and lymph nodes and IL-6 expression in DCs in the spleen of Aire knockout mice both significantly increased compared to those in the wild-type mice after H1N1 influenza virus infection. In addition, the results of the study *in vitro* also showed that the expression levels of ICOSL and IL-6 in BMDCs of Aire knockout mice both increased, and the numbers of T_{FH} cells increased. After the function of ICOSL and IL-6 was neutralized, the percentage of T_{FH} cells decreased. The study reported by Hu et al. in systemic lupus erythaematosus and rheumatoid arthritis mouse models showed that the numbers of T_{FH} cells in the spleens and lymph nodes and the number of B cells in the germinal center depended on ICOS-ICOSL interaction. After treatment using anti-ICOSL antibodies, the numbers of T_{FH} cells and germinal center B cells decreased, and the clinical symptoms of diseases were ameliorated (Hu et al., 2009). The study reported by Kim et al. showed that blocking IL-6 function using IL-6 receptor antibodies resulted in significant reductions in the numbers of T_{FH} cells in the spleens of model mice (Kim et al., 2014); which were consistent with results in this study. Therefore, it could be confirmed that Aire inhibited T_{FH} cell differentiation through the down-regulation of ICOSL and IL-6 expression in BMDCs. However, whether Aire can participate in T_{FH} cell differentiation through other mechanisms and the mechanisms underlying the regulation of ICOSL and IL-6 in BMDCs still require further in-depth studies.

This study utilized Aire knockout mice to observe the influence of Aire deficiency on T_{FH} cell differentiation. The results showed that the loss of Aire resulted in increases in the numbers of T_{FH} cells, both *in vivo* and *in vitro*. Further studies showed that Aire deficiency promoted T_{FH} cell differentiation through the upregulation of ICOSL and IL-6 expression in DCs, suggesting that Aire could inhibit ICOSL and IL-6 expression to further inhibit T_{FH} cell differentiation. This study provides a new way to fully understand the role and mechanisms of Aire in peripheral immune tolerance. In addition, this study provides experimental basis for the treatment of various autoimmune diseases through the regulation of T_{FH} cell differentiation and the production of autoantibodies using Aire as the target.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.04.007.

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