Cytotherapy, 2018; ■■: ■■–■■

International Society ISCT Cell & Gene Therapy

Therapeutic effect of human amniotic epithelial cells in murine models of Hashimoto's thyroiditis and Systemic lupus erythematosus

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Abstract

Background aims. The chronic inflammation of autoimmune diseases develops repetitive localized destruction or systemic disorders, represented by Hashimoto's thyroiditis (HT) and Systemic lupus erythematosus (SLE) respectively. Currently, there are no efficient ways to treat these autoimmune diseases. Therefore, it is critically important to explore new therapeutic strategies. The aim of this study was to investigate the therapeutic efficacy of human amniotic epithelial cells (hAECs) in murine models of HT and SLE. *Methods.* Experimental autoimmune thyroiditis (EAT) was induced in female CBA/J mice by immunization with porcine thyroglobulin (pTg). hAECs were intravenously administered at different time points during the disease course. MRL-Fas^{lpr} mice, a strain with spontaneously occurring SLE, were intravenously administered hAECs when their sera were positive for both anti-nuclear antibodies (ANAs) and anti-double-stranded DNA (anti-dsDNA) antibodies. Two weeks after the last cell transplantation, blood and tissue samples were collected for histological examination and immune system analysis. *Results.* hAECs prevented lymphocytes infiltration into the thyroid and improved the damage of thyroid follicular in EAT mice. Correspondingly, hAECs administration reduced anti-thyroglobulin antibodies (TGAb), anti-thyroid peroxidase antibodies (TPOAb) and thyroid stimulating hormone (TSH) levels. SLE mice injected with hAECs appeared negative for ANAs and anti-dsDNA antibodies and showed reduced immunoglobulin profiles. Mechanically, hAECs modulated the immune cells balance in EAT and SLE mice, by downregulating the ratios of Th17/Treg cells in both EAT and SLE mice and upregulating the proportion of B10 cells in EAT mice. This was confirmed by in vitro assay, in which hAECs inhibited the activation of EAT mice-derived splenocytes. Moreover, hAECs improved the cytokine environment in both EAT and SLE mice, by suppressing the levels of IL-17A and IFN-γ and enhancing TGF-β. *Conclusion.* These results demonstrated the immunoregulatory effect of hAECs for inflammation inhibition and injury recovery in HT and SLE murine models. The current study may provide a novel therapeutic strategy for these autoimmune diseases in clinic.

Key Words: *cell therapy, experimental autoimmune thyroiditis, human amniotic epithelial cells, Hashimoto's thyroiditis, immunomodulatory, Systemic lupus erythematosus*

Introduction

Autoimmune diseases arise from the misrecognition of immune system on the own tissues or organs. The consequent chronic inflammation develops repetitive localized destruction or systemic disorders, corresponding to Hashimoto's thyroiditis (HT) and Systemic lupus erythematosus (SLE) as representative diseases respectively. HT is an organ-specific autoimmune disease characterized by the production of thyroid-specific autoantibodies, mononuclear cell infiltration and destruction of the thyroid, resulting

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(Received 1 February 2018; accepted 2 April 2018)

ISSN 1465-3249
https://doi.org/10.1016/j.jcyt.2018.04.001

in the development of hypothyroidism [1]. On the other hand, SLE is a multisystem autoimmune disease characterized by the production of autoantibodies against diverse nuclear antigens, causing immune complexes to accumulate in multiple organs [2]. Both of the diseases arise through self-reactive response of the human immune system, in which autoantibodies derived from activated B cells and self-reactive T cells recognize self-antigens to result in tissue damages. Therefore, despite the different target organs and inflammatory processes, the two kinds of autoimmune diseases share the common immune mechanism of aberrant reaction of both T cells and B cells. The current treatment approaches aiming these autoimmune diseases includes immune inhibitors, anti-inflammatory agents, and antimalarial drugs [3]. However, the nonspecificity and one-sidedness limit the effectiveness of the treatments. Also, these medicines can lead to cumulative drug toxicity and other unexpected side effects. For example, levothyroxine (L-T4), the mostly used drug of the thyroxine replacement therapy in HT patients, may induce bone loss [4], exacerbate angina pectoris [5] and psychological illness [6]. Thus, new strategies of immunomodulation are required for more effective and safer treatment.

Stem cell therapy has been considering as an ideal option because of their long-term recovering activity for injuries. Nevertheless, the use of embryonic stem cells (ESCs) have ethical concerns; both ESCs and induced pluripotent stem cells (iPSCs) confront challenges of tumorigenicity and immunogenicity. A number of studies reported that mesenchymal stem cells (MSCs) had therapeutic effect in certain immune disease models based on their immunomodulatory properties [7–9]. However, the flexible immune response and controversial tumorigenicity under pathological environment restrict their use for autoimmune disease patients. Moreover, autologous MSCs therapy requires invasive extraction for harvesting in most of cases [10].

In contrast, cells derived from the placenta, such as human amniotic epithelial cells (hAECs), appear to have several important advantages over other stem cell lineages. A large number of hAECs (more than 100 million from a term-delivered placenta) can be noninvasively harvested without ethical concerns [11]. hAECs have none tumorigenicity due to the lack of telomerase [12] and potential immune tolerance with expression of nonclassic HLA-G and without MHC II antigens [13–15]. Importantly, hAECs demonstrated immunomodulatory properties in inhibiting the activity of immune cells [16,17], and held efficacy for the treatment of autoimmune diseases such as multiple sclerosis (MS) in vivo [18–20].

Despite the unique characteristics, hAECs have surprisingly received little attention for autoimmune disease therapy. In the present study, we examined the treatment efficacy of hAECs in HT and SLE murine models and investigated involved immunoregulatory mechanism.

Methods

Human amniotic epithelial cells isolation and culture

Human amniotic membranes were obtained with written and informed consent from healthy mothers undergoing cesarean section. The procedure was approved by the institutional patients and ethics committee of the International Peace Maternity and Child Health Hospital, Shanghai Jiao tong University School of Medicine. All donors were negative for hepatitis A, B, C, and D and HIV-I and TPAB (Treponema pallidum antibody). hAECs were isolated from the collected placenta. In brief, the amniotic membrane was peeled from the placental chorion and washed in HBSS to discard blood cells. The amniotic membrane was digested with 0.25% trypsin (EDTA) for 30 min at 37°C in a water bath. Two volumes of complete culture medium (F12/DMEM, 10% KSR (Knockout Serum Replacement), 2 mM L-glutamine, 1% nonessential amino acid, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% antibiotic-antimycotic (all from Gibco) and 10 ng/ml EGF (Peprotech)) was added to the trypsin digestion medium, and the sample was centrifuged for 10 min at 300 g. The cell pellet was then suspended in the complete culture medium for the following cell culture by incubation in 5% CO_2 in air at 37°C. The immunophenotype of the hAECs was then analyzed for HLA-ABC, HLA-DQ, HLA-DR, CD45, CD31, CD34, CD44, CD146 and isotype controls (all from eBioscience) using flow cytometry and pan-Cytokeratin, E-cadherin (all from Abcam) through immunocytochemistry. Cell viability, as estimated by a trypan blue exclusion assay, was above 95% prior to cell transplantation. Passages 1–3 of hAECs isolated from more than 3 different donors were utilized in this paper.

Animals

Female CBA/J mice ($H2^K$), a strain susceptible to EAT, were obtained from Shanghai Research Center for Model Organisms. MRL-Fas^{lpr} mice, a strain with spontaneously occurring SLE, were obtained from Nanjing Biomedical Research Institute of Nanjing University. The mice were raised under specific pathogen-free conditions with free access to food and water in Zhejiang University Laboratory Animal Center. All animal studies were approved by the University Committee on the Use and Care of Animals.

Table I. Grouping of EAT mice for time point selection of hAECs administration.

Group	First immune challenge	Second immune challenge	Treatment	Harvest
1	Day 0	Day 14	Day 14	Day 28
2	Day 0	Day 14	Day 21	Day 35
3	Day 0	Day 14	Day 0,7,14,21	Day 35
4	Day 0	Day 14	Day 35	Day 49

hAECs administration in EAT and SLE mice

EAT was induced in 8-week-old female CBA/J mice. The mice were immunized subcutaneously under 5% chloral hydrate anesthesia with 100 μg of pTg in sterile PBS emulsified in 100 μl of CFA (complete Freund's adjuvant, which contains a *Mycobacterium tuberculosis* strain at 5 mg/ml) on day 0. Two weeks later, the mice were boosted with the same dose of pTg in IFA (incomplete Freund's adjuvant, all from Sigma).

To evaluate the effect of timing of hAECs administration, the injection time points were selected based on the disease progress of EAT mice (Supplementary Figure S1D) at day 14, day 21, day 0, 7, 14, 21 or day 35. At different time points, 1.5×10^6 hAECs in 100 μl of sterile PBS or 100 μl PBS alone as a control were injected into the tail vein. The animals were sacrificed 2 weeks after the last transplantation (Table I).

When MRL-Fas^{lpr} mice sera were double positive for ANAs and anti-dsDNA antibodies around 12–16 weeks old, they were identified as SLE mice, which were then injected with 7.5×10^5 hAECs in 100 μl of sterile PBS or 100 μl PBS alone intravenously twice at the same day. The animals were sacrificed 2 weeks after the cells transplantation (Table II). At least 6 mice in each group were employed in the experiments (Normal control group, Disease group, hAECs-treated group).

Histology

Thyroids were surgically removed and fixed in 4% PFA, embedded in paraffin and sectioned using a standard method. Infiltration was evaluated on 5-μm-thick sections stained with hematoxylin and eosin (H&E). Thyroid histopathology scores were assigned based on the percentage of thyroid follicles replaced with infiltrating lymphocytes as previously described in detail [21]. The severity of thyroiditis was graded on a scale of 0–4, as follows: grade 0, normal histology; grade 1, interstitial accumulation of inflammatory cells distributed around one or two follicles; grade 2, one or more foci of inflammatory cells reaching at least the size of one follicle; grade 3, 10–40% of the thyroid replaced with inflammatory cells; grade 4, destruction of more than 40% of thyroid tissue. Thyroids tissue sections were stained for lymphocytes with rat anti-mouse CD45R (Abcam) (1:250). VECTASTAIN Elite ABC Kit (Standard*) (Vector) and AEC Peroxidase (HRP) Substrate Kit (Vector) were performed according to the manufacturer's instructions.

Table II. Experimental procedures of mice treatment for SLE analysis.

Group	Treatment (Day 0)	Harvest (Day 14)
Control	PBS	Serum and tissue
SLE	PBS	Serum and tissue
SLE + hAECs	hAECs	Serum and tissue

Serological assays

For EAT mice, TGAb, TPOAb, TSH, total triiodothyronine (TT3), and total thyroxine (TT4) levels in sera samples were assayed using commercial ELISA kits. For SLE mice, ANAs were measured by immunofluorescence using HEp-2-coated-slides (Euroimmun). Anti-dsDNA autoantibodies were measured by immunofluorescence analysis of *Crithidia luciliae* kinetoplast staining (Euroimmun). To quantify sera ANAs, anti-dsDNA antibodies, mean fluorescence intensity (MFI) was measured for ≥10 cells/sample using ImagePro Plus 6.0 software, and average MFI was determined. IgG isotypes (IgG1, IgG2a, IgG3) were measured by ELISA established using commercially available reagents.

Sera cytokine levels of IL-17A, IFN-γ, IL-4, IL-10 and TGF-β of EAT and SLE mice were measured by ELISA. All ELISA kits were from Hengyuan Biological Technologies (Shanghai, China). Specific experimental steps were performed according to the manufacturer's instructions.

Flow cytometry

Spleens were surgically removed from EAT and SLE mice. Single-cell suspensions of splenocytes were prepared, treated with RBC lysis buffer, and then cultured in complete RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 100 U/ml penicillin/streptomycin (all from Gibco).

For IL-17A staining, splenocytes were stimulated with a combination of 25 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin. After 2 h, 1.7 μg/ml monensin (all from Lianke Biotechnology, Hangzhou, China) was added, and the cells were incubated for an additional 3 h. $CD4^+IL\text{-}17A^+$ (Th17) cell or $CD4^+Foxp3^+$ (Treg) cell markers were identified using a rat anti-mouse CD4-FITC antibody, a rat anti-mouse IL-17A-PE antibody or a rat anti-mouse Foxp3-PE antibody. Intracellular cytoplasmic staining was performed using Intracellular Fixation and

Permeabilization Buffers. Intracellular nuclear staining was performed using a Foxp3 Fixation/Permeabilization Kit according to the manufacturer's protocol. $CD5^{+}CD1d^{hi}CD19^{+}$ (B10) cell markers using a rat anti-mouse CD5-FITC, a rat anti-mouse CD1d-PE, and a rat anti-mouse CD19-APC antibody. Isotype controls were used to correct for nonspecific binding (all from eBioscience). Flow cytometric analysis was used Kaluza Analysis 1.5a software.

Cytokine production in vitro

Splenocytes (1.2×10^6) of EAT mice at the peak of disease (35 day) were stimulated with pTg (80 μg/ml) in the presence or absence of hAECs (1.2×10^5). Cell-free culture supernatants were collected after 48 h by centrifugation. Cytokine levels of IL-17A, IFN-γ, IL-4, IL-10 and TGF-β were measured by ELISA (Hengyuan Biological Technologies, Shanghai, China).

Statistical analysis

Statistical significance was determined with GraphPad Prism 7 software. Differences between two experimental groups were determined using an unpaired with Welch's correction, two-tailed t test. Differences among three experimental groups were determined using one-way analysis of variance with Tukey's multiple comparison test. Confidence intervals of 95% were deemed significant.

Results

hAECs characterization

The hAECs prepared in this study were cuboidal with 5–10 μm diameters, which is the typical appearance for epithelial cells (Figure 1A). The hAECs showed a homogeneous population and nearly all cells showed persistent expression of pan-Cytokeratin (Figure 1B) and E-cadherin (Figure 1C), which are specific markers of epithelial cells. For immunophenotypic characterization of hAECs, surface protein expression was examined by flow cytometry. The hAECs were negative for the MSC markers CD44, CD146 (Figure 1H), endothelial markers CD31, CD34 (Figure 1D), and the hematopoietic lineage markers: CD45(Figure 1E). hAECs were positive for MHC-Ia: HLA-ABC (Figure 1F) and negative for MHC-II: HLA-DR or HLA-DQ (Figure 1G).

hAECs suppressed the development of HT in EAT mice

To determine whether hAECs have therapeutic effect on HT, murine EAT model was established (Supplementary Figure S1) and hAECs were administrated by tail-vein injection on different time points of the EAT duration as indicated (Table I). The sera levels of HT diagnosis markers were examined. Compared with non-induced control mice, remarkably increased TGAb, and TPOAb, and TSH were detected in EAT mice (Supplementary Figure S1E-G). In contrast, the augmented levels of these markers were considerably reversed when hAECs were administered at any injection time points (Figure 2H-J). These findings indicated hAECs attenuated the thyroid-specific autoimmune response induced by thyroglobulin immunity.

On the other hand, lymphocytes infiltration and thyroid follicular destruction based histological evaluation was determined in different groups of mice thyroid. Compared with the EAT groups, hAECs injection on day 35 significantly suppressed the lymphocytes infiltration into the thyroid as evidenced by the pathology quantification (Figure 2D with representative images in Figure 2E) and confirmed by immunochemical staining of CD45R (Figure 2F, G), while a decreased trend was observed in the mice with hAECs administration at other time points (Figure 2A-C). Due to a better rescue effect of hAECs on EAT with injection on day 35, the peak of EAT development, we focused on this time point for further mechanism analysis (Table III).

hAECs suppressed the SLE development in lupus-prone mice

The investigation of the effect of hAECs on SLE was determined in lupus-prone MRL-Fas^{lpr} mice (SLE mice). Since the signature symptom of SLE is the generation of large amount of ANAs against both RNA-containing and DNA-containing autoantigens, a fluorescent ANAs assay was performed. The sera of the control mice did not recognize the autoantigens in human laryngeal carcinoma epithelial (HEp-2) cells

Table III. Experimental procedures of mice treatment for EAT analysis.

Group	First immune challenge (Day 0)	Second immune challenge (Day 14)	Treatment (Day 35)	Harvest (Day 49)
Control	PBS	PBS	PBS	Serum and tissue
EAT	pTg + CFA	pTg + IFA	PBS	Serum and tissue
EAT + hAECs	pTg + CFA	pTg + IFA	hAECs	Serum and tissue

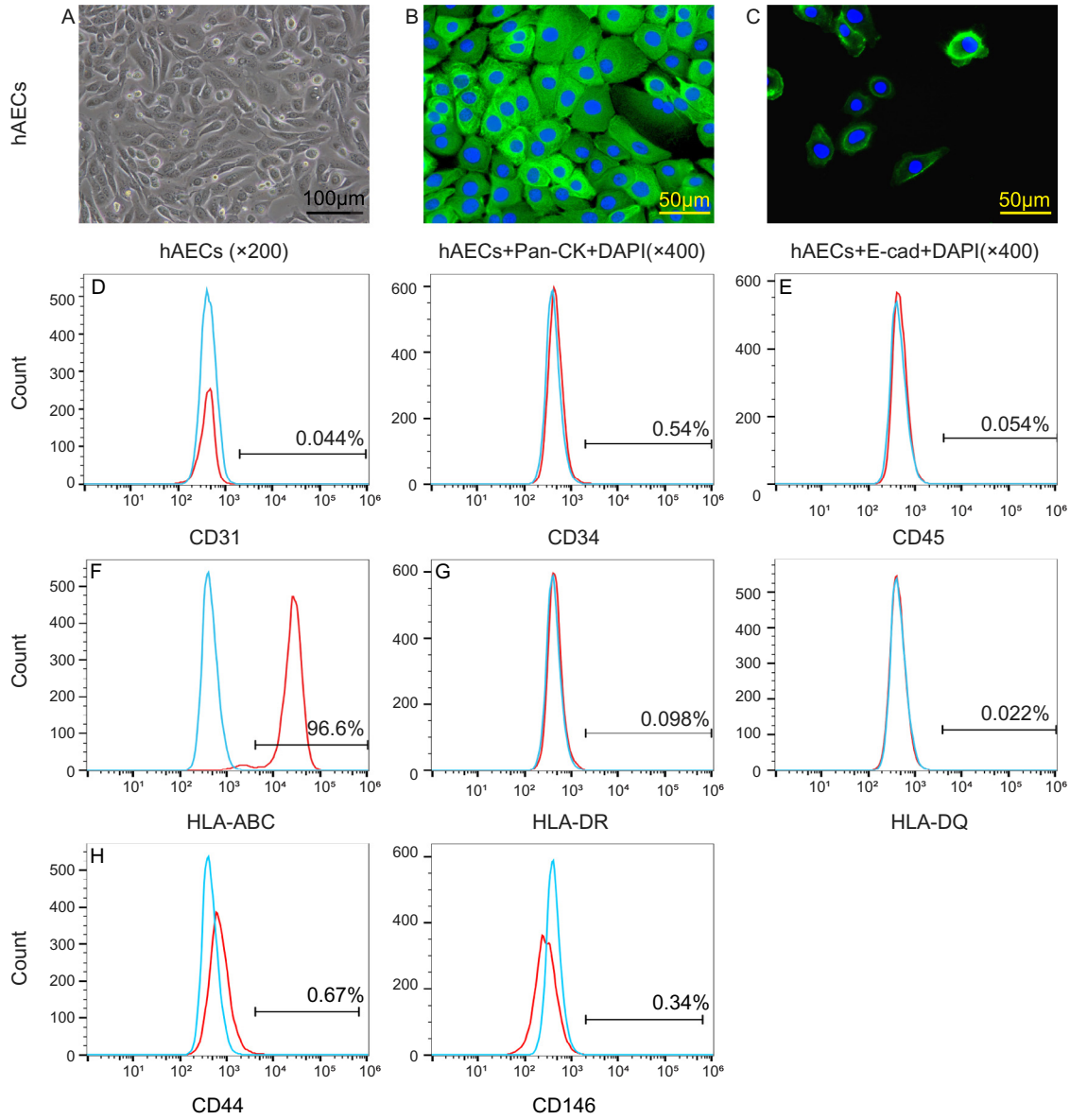

Figure 1. Characteristics of hAECs. The isolated hAECs showed a homogeneous population of cuboidal cells with 5–10 μm diameters (**A**). Nearly all cells showed persistent expression of pan-Cytokeratin (**B**) and E-cadherin (**C**), which are specific markers of epithelial cells. Flow cytometry histogram for surface markers demonstrated that hAECs were positive for HLA-ABC (**F**) and negative for CD31, CD34 (**D**), CD45 (**E**), HLA-DR, HLA-DQ (**G**), and CD44, CD146 (**H**). Data are presented as the mean ± SD from 3 separate specimens from three independent experiments. hAECs, human amniotic epithelial cells; Pan-CK, pan-Cytokeratin; E-cad, E-cadherin; CD, cluster of differentiation; HLA, human leukocyte antigen.

as evidenced by negative fluorescent staining (Figure 3A). In contrast, sera of typical SLE mice produced a homogenous nuclear ANAs pattern with abundant positive staining (Figure 3B). However, after hAECs administration, the sera ANAs response almost disappeared (Figure 3C, D). The repression of autoantibodies by hAECs was further confirmed by a high-specificity immunofluorescence staining for detecting antibodies against dsDNA in *Crithidia luciliae* kinetoplast (Figure 3E-H). Also, MRL-Fas^{lpr} mice develop an age-dependent increase in sera circulating immunoglobulins during the SLE progression. Consistently, hAECs injection could reverse the development of hypergammaglobulinemia (Figure 3I-K).

hAECs preserved immune cells balance in EAT and SLE mice

To determine the immunomodulatory function of hAECs, the proportions of immune cells in the spleens of EAT and SLE mice were examined 2 weeks after hAECs administration. Compared with control mice,

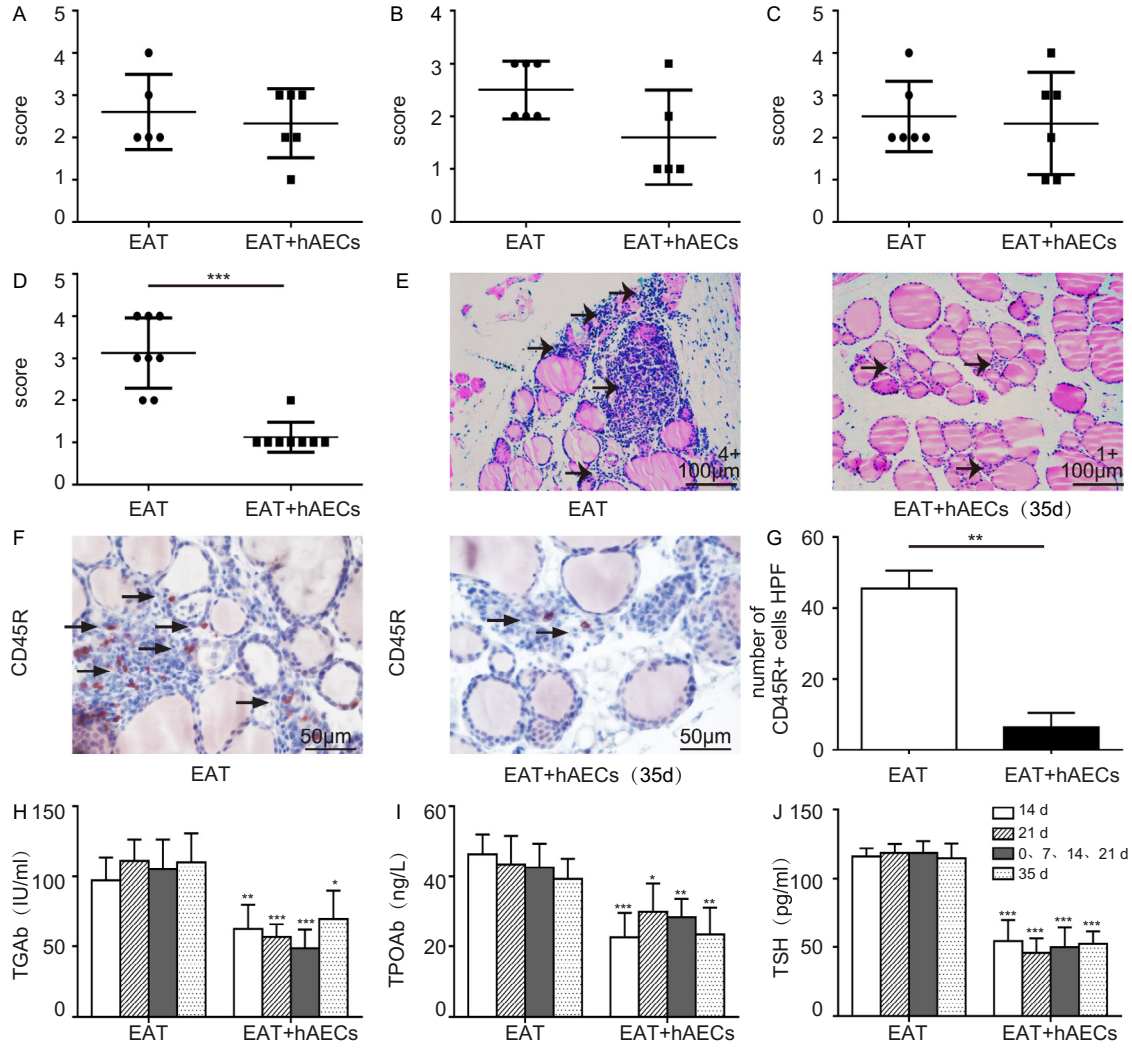

Figure 2. hAECs suppressed the development of HT in EAT mice. EAT mice were administered 1.5×10^6 hAECs at different times as indicated in Table I. Two weeks after the last cell transplantation, thyroid tissues and sera were collected from the EAT mice. Histological examination was performed based on H&E staining. Pathological scores for every mouse were evaluated when hAECs administered on day 14 (**A**), day 21 (**B**), days 0, 7, 14, and 21 (**C**), day 35 (**D**). Pathological scores for individual mice are indicated by dots. The horizontal lines represent the median severity in each group. Representative images of H&E staining in EAT mice (harvested on day 49) and EAT mice with hAECs injection at the peak of disease on day 35 (harvested on day 49) are shown in **E**. CD45R immunohistochemical staining were performed in the thyroids from EAT mice (harvested on day 49) and EAT mice with hAECs injection at the peak of disease on day 35 (harvested on day 49). Representative images are shown in **F** with quantification in **G.** The arrows indicated infiltrated mononuclear cells and $CD45R^+$cells in **E** and **F** respectively. The levels of TGAb (**H**), and TPOAb (**I**), and TSH (**J**) in EAT mice and EAT mice with hAECs were determined by ELISA. Data are presented as the mean ± SD from 5–8 separate specimens per group from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. Statistical analysis was performed using an unpaired t text with Welch's correction.

the proportion of Th17 cells in the EAT mice remarkably increased, and the augment of Th17 cells was significantly suppressed in hAECs-injected group (Figure 4A). The proportion of Treg cells was not affected by the hAECs administration (Figure 4B). On the other hand, the reduced proportion of B10 cells in the EAT mice was restored upon hAECs administration (Figure 4C). These results suggested that hAECs treatment altered the Th17/Treg cells balance by down-regulating Th17 cells and modulated the immune system via B10 cells up-regulation in EAT mice. In SLE mice, hAECs strikingly decreased the proportion of Th17 cells (Figure 5A), and enhanced the proportion of Treg cells (Figure 5B), but the proportion of B10 cells was little affected (Figure 5C). These results suggested that, different from in EAT mice, hAECs treatment altered the Th17/Treg cells balance by down-regulating Th17 cells and up-regulating Treg cells synergistically, while had no significant influence on B10 cells in SLE mice.

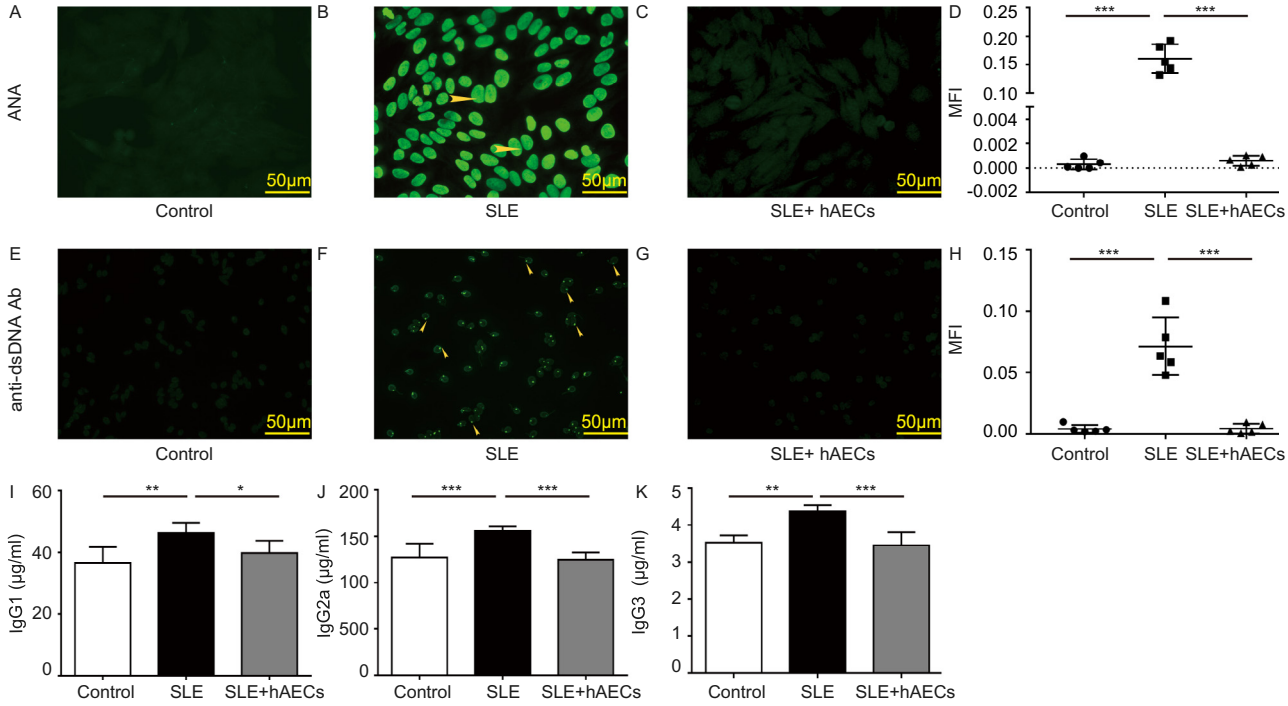

Figure 3. hAECs suppressed the SLE development in lupus-prone mice. SLE mice were injected with 7.5×10^5 hAECs intravenously twice at the same day. Two weeks after hAECs administration, immunofluorescence analysis of ANAs and anti-dsDNA antibodies, and ELISA analysis of IgG subtypes in the sera of MRL-Fas^{lpr} mice were performed. Representative images of ANAs detection in HEp-2 cells for control mice (**A**), SLE mice (**B**), and SLE mice with hAECs (**C**) are shown. Mean MFI value of ANAs obtained from individual mice were quantified in **D**. Representative images of anti-dsDNA antibodies detection in *Crithidia luciliae* kinetoplast for control mice (**E**), SLE mice (**F**), and SLE mice with hAECs (**G**) are shown. Mean MFI value of anti-dsDNA antibodies obtained from individual mice were quantified in **H**. Quantification of IgG1(**I**), IgG2a (**J**), IgG3 (**K**) were determined by ELISA. Arrows, nucleus in **B** and kinetoplast in **F**. Data are presented as the mean ± SD from 5 separate specimens per group from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. Data were compared using a one-way analysis of variance and Tukey's Multiple Comparison Test.

hAECs inhibited activation of splenocytes of EAT mice in vitro

To determine the immunoregulatory activity of hAECs in vitro, splenocytes were isolated from EAT mice at the peak of disease (day 35) and then stimulated by pTg with or without hAECs coculture. Cytokines of T cell subtypes in the splenocytes were then examined. hAECs down-regulated the production of the Th1 cytokine IFN-γ (Figure 6A) and the Th17 cytokine IL-17A (Figure 6B). There was no significant difference in IL-4 in the presence or absence of hAECs (Figure 6C). TGF-β and IL-10 were not detected in the supernatant. These results indicated that hAECs could inhibit autoimmune cell activation in vitro.

hAECs improved cytokine environment in EAT and SLE mice

Moreover, the composition of cytokine environment in EAT mice and SLE mice were analyzed with or without hAECs administration. EAT and SLE mice injected with hAECs exhibited significantly down-regulated production of the proinflammatory Th17 cytokine IL-17A (Figure 7A) and the Th1 cytokine IFN-γ (Figure 7B); however, the level of the Th2 cytokine IL-4 (Figure 7C) and IL-10 (Figure 7D) did not change. On the other hand, hAECs induced secretion of the anti-inflammatory molecule TGF-β (Figure 7E). Therefore, hAECs helped to improve the cytokine environment in the EAT and SLE mice.

Discussion

In the current study, we examined the potential effect of hAECs on HT and SLE. hAECs transplantation in EAT and spontaneous lupus mice systematically preserved organ function, reduced inflammation and altered the immune balance. To our surprise, hAECs demonstrated best treatment effect when they were administrated at the peak of diseases, although the disease phenotypes were also suppressed upon the injection of hAECs at earlier windows. These results indicated that hAECs may ameliorate both the incidence and development of EAT and spontaneous lupus. More importantly, hAECs could be ideal candidate for HT and SLE treatment in clinic, in which most patients are diagnosed with obvious symptoms. To our knowledge, this is the first study to report

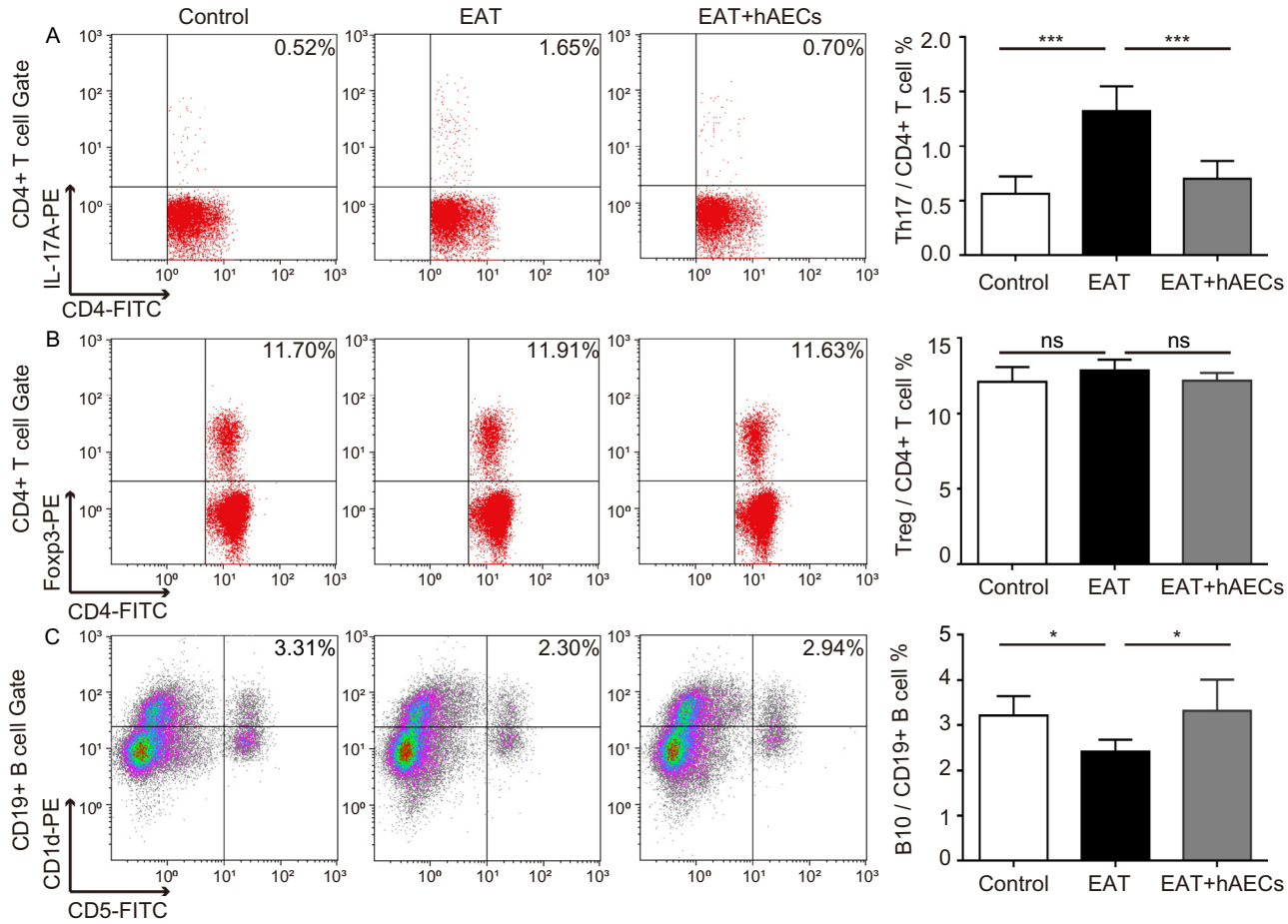

Figure 4. The effect of hAECs administration on proportions of immune cells in EAT mice. Spleens were harvested on day 49 from non-diseased control mice, EAT mice and EAT mice with hAECs injection at the peak of disease (day 35). Th17 (**A**), Treg (**B**), and B10 (**C**) cells proportions were determined by flow cytometry. Data are presented as the mean ± SD from 5–8 separate specimens per group from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data were compared using a one-way analysis of variance and Tukey's Multiple Comparison Test. Representative flow scatter diagrams and quantifications are shown.

the therapeutic benefit of hAECs in these autoimmune diseases. The key function of hAECs in EAT and spontaneous lupus is the immunomodulatory effect. Correspondingly, the regulation of immune system by hAECs was further investigated.

In the present study, although hAECs improved disease symptoms in EAT and spontaneous lupus, they exhibited different modulatory styles on immune balance. This refers to both B and T cells, in which B10 cells are the major target B cells. As reported, the proportion of B10 cells decreases and is maintained at a lower level during the development of thyroiditis [22]. The infusion of splenic B10 cells significantly prolonged the survival of NZB/W auto-immune mice [23]. Our results demonstrated that hAECs selectively upregulated B10 cells in EAT mice but had little effect on B10 population in SLE mice. This can be due to different behaviors of B10 cells in different autoimmune diseases: B10 cells number is significantly augmented in lupus-prone mice, but below normal levels in susceptible to exogenous autoantigen-induced autoimmune disease [24]. Noticeably, B10 cells are the predominant source of B cell-produced IL-10 [25], so that work as regulatory B cells to inhibit the differentiation of Th17 cells [26].

Indeed, Th17 cells population was downregulated in EAT mice upon hAECs administration. Consistent with previous studies, our observation confirmed that Th17 cells played a critical role in the pathogenesis of HT [27]. Importantly, hAECs exerted their immunoregulatory function in EAT mice partially by suppressing the Th17 proportion and Th17-derived IL-17A, so that inhibited the further secretion of inflammatory cytokines and the recruitment of inflammatory cells in the thyroid glands. On the other hand, IL-17A also appears to participate in the development of several SLE complications. The expression of IL-17A is elevated in both SLE patients and in lupus-prone mice, and it is positively correlated with disease activity [28]. Similarly,

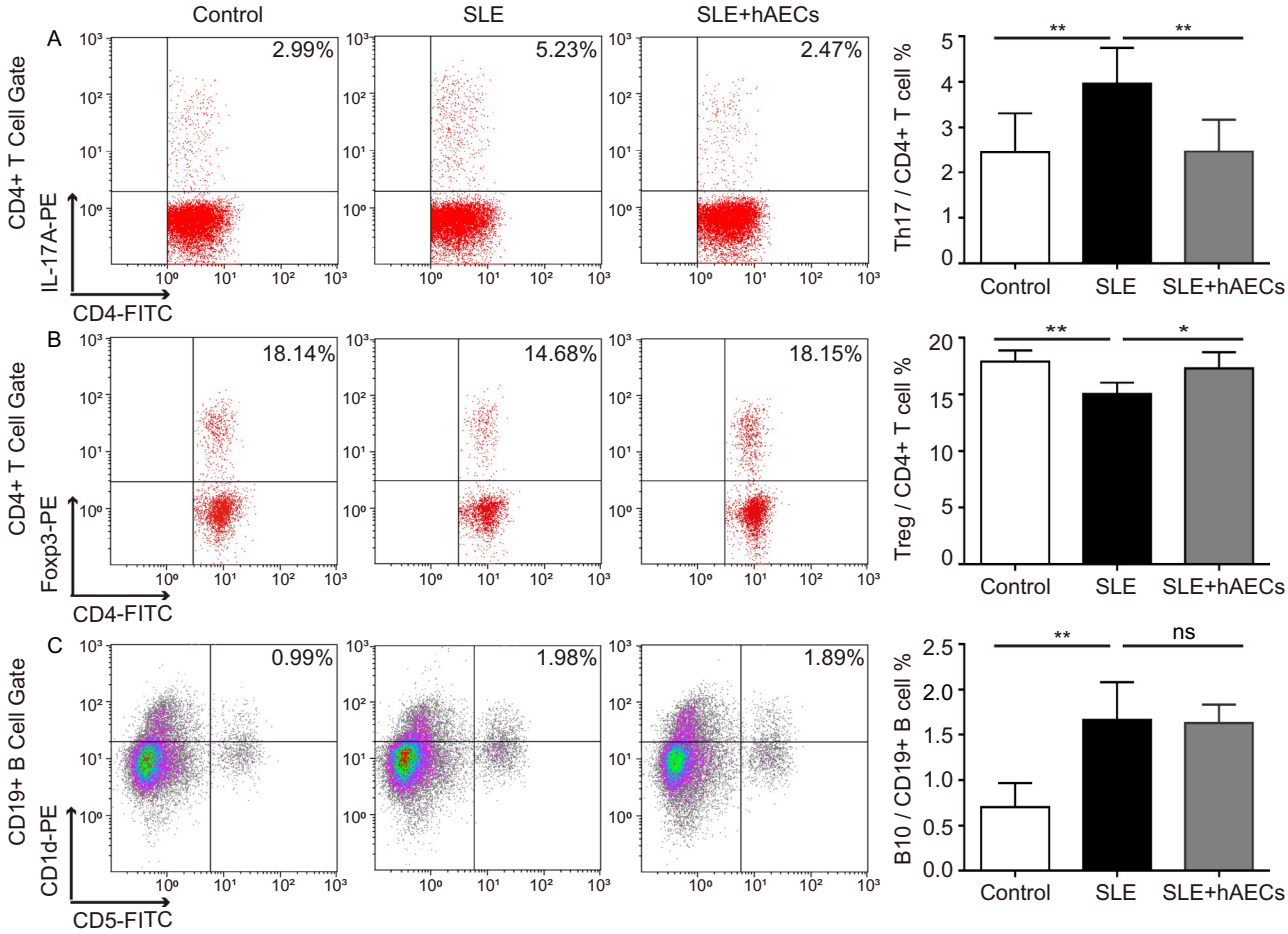

Figure 5. The effect of hAECs administration on proportions of immune cells in SLE mice. SLE mice were injected with 7.5×10^5 hAECs intravenously twice at the same day. Two weeks after hAECs administration, spleens were harvested from non-diseased control mice and SLE mice with or without hAECs treatment. Th17 (**A**), Treg (**B**), and B10 (**C**) cells proportions were determined by flow cytometry. Data are presented as the mean ± SD from 5 separate specimens per group from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data were compared using a one-way analysis of variance and Tukey's Multiple Comparison Test. Representative flow scatter diagrams and quantifications are shown.

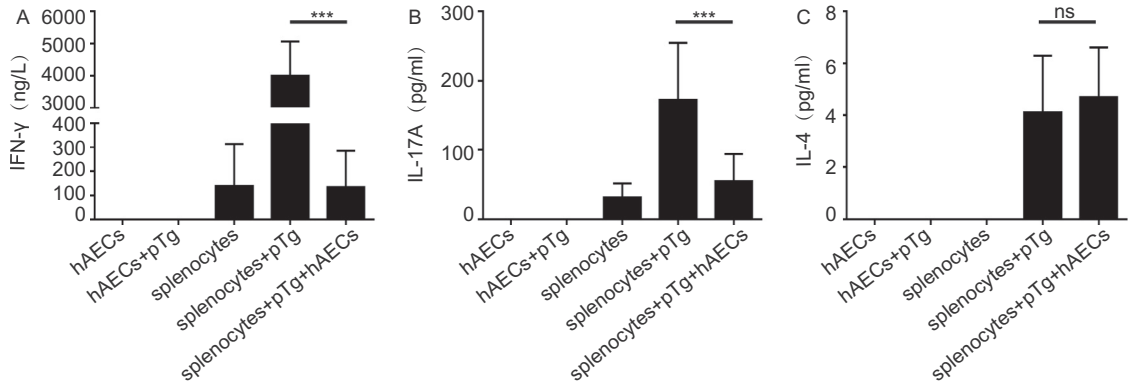

Figure 6. hAECs inhibited activation of splenocytes of EAT mice in vitro. Splenocytes isolated from EAT mice at the peak of disease (day 35) were stimulated with pTg (80 μg/ml) in the presence or absence of hAECs. The levels of the cytokines of T cell subtypes IFN-γ (**A**), IL-17A (**B**), IL-4 (**C**) in the culture supernatant were determined 48 h later by ELISA. Data are presented as the mean ± SD from 6 separate specimens per group from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data were compared using a one-way analysis of variance and Tukey's Multiple Comparison Test.

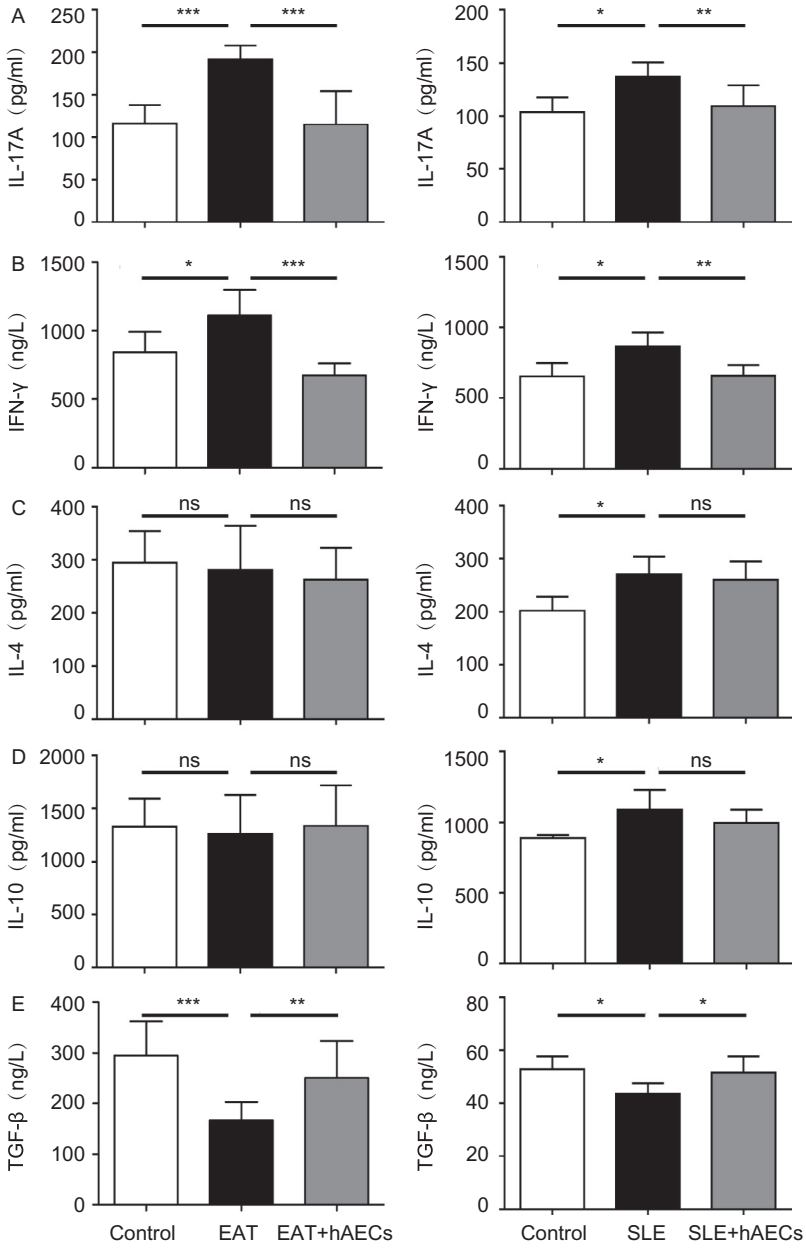

Figure 7. The effect of hAECs administration on cytokine environment in EAT and SLE mice. Sera were harvested on day 49 from non-diseased control mice, EAT mice, EAT mice with hAECs injection at the peak of disease (day 35), and on day 14 from non-diseased control mice, SLE mice, SLE mice with hAECs injection on day 0. The levels of the cytokines IL-17A (**A**), IFN-γ (**B**), IL-4 (**C**), IL-10 (**D**), and TGF-β (**E**) were then determined by ELISA. Data are presented as the mean ± SD from 6 separate specimens per group from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data were compared using a one-way analysis of variance and Tukey's Multiple Comparison Test.

decreased Th17 and IL-17A levels were detected in SLE mice after hAECs administration. However, the underlying mechanism could be based on the IL-17A-controlled B cells survival and their differentiation into immunoglobulin-secreting cells [29], as evidenced by reduced IgG profiles in SLE mice with hAECs administration.

Compared to the negative regulation of Th17 cells, hAECs administration preserved the proportion of Treg cells in SLE mice specifically, which is similar as the case of hAECs therapy on remitted MS [18]. Therefore, hAECs may correct the auto-immune systems in these diseases in different patterns. In EAT, hAECs restored B10 cells and the Th17/Treg cells balance by decreasing the Th17 cells proportion. In SLE, though the proportion of B10 cells did not change after hAECs injection in our study, the proportion of Treg cells was significantly increased, indicating that hAECs

restored the Th17/Treg cells balance by decreasing the Th17 cells proportion and increasing the Treg cells proportion. The identification of different immunoregulatory functions of hAECs in autoimmune diseases may help to develop correlated cell therapy in clinic.

Corresponding to the modulation of the immune balance by hAECs, resultant restoration of cytokine levels directly correlates to the alleviation of the symptoms of auto-immune disease. It is clear that both apoptosis and the recruitment of inflammatory cells to the thyroid in HT are under the tight control of the inflammatory cytokine microenvironment [30]. Although HT is an organ-specific autoimmune disease, it can influence the global generation of proinflammatory cytokines [31]. On the other hand, it has been established that T-cell abnormalities and aberrant T helper cytokine profiles play an essential role in the development of SLE, despite the debate regarding the dominance of Th1 or Th2 cytokines in SLE pathogenesis persists [32]. Typically, the inhibition of IFN-γ and IL-17A but augment of TGF-β by hAECs administration contribute significantly to restrain the proliferation and activation of lymphocytes.

Mechanically, hAECs restored the immune balance in these autoimmune disease models by expressing different groups of mediators, one of their major characteristics as indicated by our and other's studies [33–35]. For cell therapy, it is still debated about the predominant effect between the local cell-contact interaction and the systemic regulation of the infused cells. In the current study, we think that hAECs exerted the immunomodulatory actions in both ways. The surface expression of HLA-G, complement inhibitory proteins, CD59 antigen and decay accelerating factor could be the key factors for immunosuppression of T helper cells [15,36,37], while the presence of FasL could be responsible for induction the apoptosis of T helper cells in a cell-to-cell contact pattern in thyroid [38]. And the secretion of tissue inhibitor of matrix metalloproteinases (TIMP-1-4), IL-10, TGF-β and macrophage migration inhibiting factor (MIF) played the important role in inhibiting the proliferation and differentiation of Th1, Th2 and Th17 cells but upregulating the Treg and B10 cells systematically [16,17].

Therefore, hAECs administration could be considered as a novel strategy to treat HT and SLE. It was reported that hAECs treatment ameliorated MS, either effective on its progression or its relapse [18,19]. Further pre-clinical studies are required to determine the effect of hAECs on EAT or SLE relapse after the termination of drug treatment, and also the optimal doses, long-term therapeutic efficacy as well as the safety of multiple injections.

Acknowledgments

This work was supported by the Project of Health Collaborative Innovation of Guangzhou City (Grant No. 201704020214), the National Natural Science Foundation of China (Grant No. 81770444 and 81600354) and Fundamental Research Funds for the Central Universities of China.

Disclosure of interests: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

[1] Hasham A, Tomer Y. Genetic and epigenetic mechanisms in thyroid autoimmunity. Immunol Res 2012;54:204–13.
[2] Grammatikos AP, Tsokos GC. Immunodeficiency and autoimmunity: lessons from Systemic lupus erythematosus. Trends Mol Med 2012;18:101–8.
[3] Yildirim-Toruner C, Diamond B. Current and novel therapeutics in the treatment of Systemic lupus erythematosus. J Allergy Clin Immunol 2011;127:303–12, quiz 13–4.
[4] Christy AL, D'Souza V, Babu RP, Takodara S, Manjrekar P, Hegde A, et al. Utility of C-terminal telopeptide in evaluating levothyroxine replacement therapy-induced bone loss. Biomark Insights 2014;9:1–6.
[5] Kahaly GJ. Cardiovascular and atherogenic aspects of subclinical hypothyroidism. Thyroid 2000;10:665–79.
[6] Saravanan P, Chau WF, Roberts N, Vedhara K, Greenwood R, Dayan CM. Psychological well-being in patients on 'adequate' doses of L-thyroxine: results of a large, controlled community-based questionnaire study. Clin Endocrinol (Oxf) 2002;57:577–85.
[7] Chang JW, Hung SP, Wu HH, Wu WM, Yang AH, Tsai HL, et al. Therapeutic effects of umbilical cord blood-derived mesenchymal stem cell transplantation in experimental lupus nephritis. Cell Transplant 2011;20:245–57.
[8] Choi EW, Shin IS, Park SY, Yoon EJ, Kang SK, Ra JC, et al. Characteristics of mouse adipose tissue-derived stem cells and therapeutic comparisons between syngeneic and allogeneic adipose tissue-derived stem cell transplantation in experimental autoimmune thyroiditis. Cell Transplant 2014;23:873–87.
[9] Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in Mrl/Lpr mice. Blood 2001;97:3292–9.
[10] Broughton BR, Lim R, Arumugam TV, Drummond GR, Wallace EM, Sobey CG. Post-stroke inflammation and the potential efficacy of novel stem cell therapies: focus on amnion epithelial cells. Front Cell Neurosci 2012;6:66.
[11] Insausti CL, Blanquer M, Bleda P, Iniesta P, Majado MJ, Castellanos G, et al. The amniotic membrane as a source of stem cells. Histol Histopathol 2010;25:91–8.
[12] Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial cells. Stem Cells 2005;23:1549–59.
[13] Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U. Stem cells derived from human fetal membranes display multilineage differentiation potential. Biol Reprod 2007;77:577–88.
[14] Bilic G, Zeisberger SM, Mallik AS, Zimmermann R, Zisch AH. Comparative characterization of cultured human term

amnion epithelial and mesenchymal stromal cells for application in cell therapy. Cell Transplant 2008;17:955–68.
[15] Strom SC, Gramignoli R. Human amnion epithelial cells expressing Hla-G as novel cell-based treatment for liver disease. Hum Immunol 2016;77:734–9.
[16] Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea 2000;19:348–52.
[17] Li H, Niederkorn JY, Neelam S, Mayhew E, Word RA, McCulley JP, et al. Immunosuppressive factors secreted by human amniotic epithelial cells. Invest Ophthalmol Vis Sci 2005;46:900–7.
[18] Liu YH, Chan J, Vaghjiani V, Murthi P, Manuelpillai U, Toh BH. Human amniotic epithelial cells suppress relapse of corticosteroid-remitted experimental autoimmune disease. Cytotherapy 2014;16:535–44.
[19] Liu YH, Vaghjiani V, Tee JY, To K, Cui P, Oh DY, et al. Amniotic epithelial cells from the human placenta potently suppress a mouse model of multiple sclerosis. PLoS ONE 2012;7:e35758.
[20] McDonald CA, Payne NL, Sun G, Moussa L, Siatskas C, Lim R, et al. Immunosuppressive potential of human amnion epithelial cells in the treatment of experimental autoimmune encephalomyelitis. J Neuroinflammation 2015;12:112.
[21] Batteux F, Tourneur L, Trebeden H, Charreire J, Chiocchia G. Gene therapy of experimental autoimmune thyroiditis by in vivo administration of plasmid DNA coding for fas ligand. J Immunol 1999;162:603–8.
[22] Shi L, Bi M, Yang R, Zhou J, Zhao S, Fan C, et al. Defective expression of regulatory B cells in iodine-induced autoimmune thyroiditis in non-obese diabetic H-2(H4) mice. J Endocrinol Invest 2014;37:43–50.
[23] Watanabe R, Ishiura N, Nakashima H, Kuwano Y, Okochi H, Tamaki K, et al. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: Cd19 and B10 cell deficiency exacerbates systemic autoimmunity. J Immunol 2010;184:4801–9.
[24] Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing Il-10 (B10 cells) requires antigen receptor diversity and Tlr signals. J Immunol 2009;182:7459–72.
[25] Yang M, Deng J, Liu Y, Ko KH, Wang X, Jiao Z, et al. Il-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. Am J Pathol 2012;180:2375–85.
[26] Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique Cd1dhicd5+ phenotype controls T cell-dependent inflammatory responses. Immunity 2008;28:639–50.
[27] Horie I, Abiru N, Nagayama Y, Kuriya G, Saitoh O, Ichikawa T, et al. T helper type 17 immune response plays an indispensable role for development of iodine-induced autoimmune thyroiditis in nonobese diabetic-H2h4 mice. Endocrinology 2009;150:5135–42.
[28] Qian W, La Cava A. Il-17 in Systemic lupus erythematosus. Clin Invest 2012;2:417–21.
[29] Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont MC, Ranchin B, et al. Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009;10:778–85.
[30] Ganesh BB, Bhattacharya P, Gopisetty A, Prabhakar BS. Role of cytokines in the pathogenesis and suppression of thyroid autoimmunity. J Interferon Cytokine Res 2011;31:721–31.
[31] Choi EW, Shin IS, Bhang DH, Lee DH, Bae BK, Kang MS, et al. Hormonal change and cytokine mrna expression in peripheral blood mononuclear cells during the development of canine autoimmune thyroiditis. Clin Exp Immunol 2006;146:101–8.
[32] Hayashi T, Hasegawa K, Sasaki Y, Mori T, Adachi C, Maeda K. Systemic administration of interleukin-4 expressing plasmid DNA delays the development of glomerulonephritis and prolongs survival in lupus-prone female Nzb X Nzw F1 mice. Nephrol Dial Transplant 2007;22:3131–8.
[33] Jiawen S, Jianjun Z, Jiewen D, Dedong Y, Hongbo Y, Jun S, et al. Osteogenic differentiation of human amniotic epithelial cells and its application in alveolar defect restoration. Stem Cells Transl Med 2014;3:1504–13.
[34] Yao X, Guo Y, Wang Q, Xu M, Zhang Q, Li T, et al. The paracrine effect of transplanted human amniotic epithelial cells on ovarian function improvement in a mouse model of chemotherapy-induced primary ovarian insufficiency. Stem Cells Int 2016;2016:4148923.
[35] Banas RA, Trumpower C, Bentlejewski C, Marshall V, Sing G, Zeevi A. Immunogenicity and immunomodulatory effects of amnion-derived multipotent progenitor cells. Hum Immunol 2008;69:321–8.
[36] Rooney IA, Morgan BP. Protection of human amniotic epithelial cells (Haec) from complement-mediated lysis: expression on the cells of three complement inhibitory membrane proteins. Immunology 1990;71:308–11.
[37] Rooney IA, Morgan BP. Characterization of the membrane attack complex inhibitory protein Cd59 antigen on human amniotic cells and in amniotic fluid. Immunology 1992;76:541–7.
[38] Surendran S. Possible role of Fas antigen (Cd 95) in human amniotic epithelial cell death: an in vitro study. Cell Biol Int 2001;25:485–8.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2018.04.001.