Original Article
Mechanistic study of Erianin in alopecia areata

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Abstract: Objective: To investigate the role and mechanisms of Erianin in treating alopecia areata. Methods: A C3H/HeJ AA mouse model was established using skin transfer or adoptive T-cell transfer. All mice received either systemic or topical treatments. Photographic documentation was used to monitor hair growth, which was quantified using G*Power software. Infiltrating inflammatory markers in the skin were assessed using immunofluorescence staining, and the infiltrating immune cell populations in the skin and subcutaneous draining lymph nodes (SDLNs) were analyzed using flow cytometry. Results: Erianin effectively inhibited the function of effector T cells, significantly suppressing Alopecia Areata (AA) in C3H/HeJ transplanted mice. It also reversed systemic manifestations of AA and effectively promoted hair growth in AA mice. Conclusion: Erianin demonstrates a potent therapeutic effect on AA, primarily through modulation of T cell immune function.

Keywords: Erianin, alopecia areata (AA), mechanism, immune regulation

Introduction

Alopecia areata (AA) is a sudden-onset, non-scarring disease characterized by round or oval patches of hair loss, with the scalp being the most common site of onset. Hair loss may progress rapidly, leading to complete scalp hair loss (alopecia totalis, AT) within days or months. In severe cases, patients may also lose eyebrow, eyelash, armpit, and pubic hair, leading to alopecia universatis (AU) [1, 2]. The incidence of alopecia is rising, likely due to societal, occupational, and lifestyle changes, which significantly impact patients' appearance and psychological well-being, triggering psychological issues such as depression and anxiety [3, 4]. Additionally, AA is often associated with autoimmune and inflammatory conditions, including autoimmune thyroiditis, vitiligo, systemic lupus erythematosus, rheumatoid arthritis, pernicious anemia, psoriasis, atopic diseases (dermatitis, asthma, and rhinitis), psychiatric disorders (anxiety, eating disorders, and obsessive-compulsive disorder), zinc deficiency, vitamin D deficiency, and metabolic syndrome [5, 6].

The exact pathobiology of AA has remained elusive, and therapeutic measures are still limited at present. It is widely accepted that this AA is an autoimmune disease, influenced by both genetic predisposition and triggering factors. Additionally, non-autoimmune factors such as [7, 8] neuropsychiatric aspects, immune system irregularities, cytokines, endocrine disorders, and genetic elements are believed to be involved. Factors such as serum inflammatory components in the microcirculation of the hair follicle and vascular endothelial cell growth factors are thought to play a crucial role in the development of AA.

AA is associated with the loss of immune privilege in the hair follicle and an increase in auto-immune-mediated destruction of follicles. Local inflammation in the hair follicle or the abnormal presence of follicular autoantigens can trigger an autoimmune response by self-reactive CD8+ T cells. This response triggers the release of γ-interferon (IFN-γ) or substance P, disrupting the immune privilege of hair follicles and initiating a series of inflammatory reactions that ulti-

mately lead to hair loss. Many immune cells and factors are involved in the pathogenesis of AA. IFN-γ plays a key role in the collapse of immune privilege by upregulating the expression of MHC class I and II molecules in the follicles [9, 10]. During the active phase of AA, CD4+ and CD8+ T cells, including perifollicular lymphocytes, are present around the hair follicle in the anagen phase. Over time, these immune cells infiltrate the keratin producing cells of the hair matrix, causing tissue damage and inducing hair matrix apoptosis [11]. CD4+CD25+ regulatory T cells play a crucial role in modulating immune responses, primarily by secreting various inhibitory cytokines, which in turn influence the differentiation of Th1 and Th2 cells [12].

Chinese medicine has long been known for its effectiveness in preventing and treating hair loss [13]. Chinese herbal remedies not only promote hair growth and restoration, but also regulate abnormal hair follicle growth through multiple pathways, targets, and mechanisms [14]. However, current research on natural medicines for hair growth is limited, focusing primarily on remedies such as *chasteberry, Polygonum multiflorum*, and *Ligusticum chuanxiong Hort rhizome* [15]. Erianin, a bibenzyl analog found in *Dendrobium*, possesses a wide range of pharmacological effects and has been found to play a therapeutic role in various conditions, including cancers, diabetic retinopathy, and ulcerative colitis [16]. MO et al. [17] demonstrated that Erianin inhibited the proliferation of HaCaT keratinocytes and increased the level of reactive oxygen species (ROS) in these cells. In addition, Erianin promotes apoptosis of HaCaT cells through the ROS-mediated JNK/c-Jun signaling pathway, thereby inhibiting the development of psoriasis. Although AA is not life-threatening, it significantly reduces patients' quality of life, often to a greater extent than psoriasis or atopic dermatitis [18]. Therefore, this study aims to explore the therapeutic effects and underlying mechanisms of Erianin in treating AA.

Materials and methods

Mice

Male C3H/HeJ mice that were aged 6-8-weeks and weighed around 20 g were provided by The First Hospital of Hebei Medical University and housed in a specific pathogen-free (SPF) barrier environment, and all experiments followed the provisions of the institutional guidelines approved by the First Hospital of Hebei Medical University. Institutional Animal Care and Use Committee.

AA mouse model

The AA model was established using well-established methods [19]. Mice were randomized to control and different treatment groups, with 5 mice in each group. In brief, mice were anesthetized by intraperitoneal injection of amobarbital (30 mg/kg body weight). Then, a 1 cm diameter piece of skin from a spontaneously AA-affected C3H/HeJ mouse was transplanted to a healthy C3H/HeJ recipient mouse. Alternatively, CD8+ T cells were isolated from the subcutaneous draining lymph nodes (SDLNs) of spontaneous C3H/HeJ AA mice using magnetic beads and injected into recipient mice. Hair status of the mice was monitored and photographed weekly.

In vivo treatment

Erianin, provided by Shanghai Yuanye Biotechnology Co., was stored as a 10 mM master stock in DMSO. In the AA prevention study, erianin was administered via intraperitoneal injection (250 μg per mouse) twice a week for 6-8 weeks, commencing from the day of skin transplantation or adoptive transfer of T-cells. In the AA reversal study, erianin treatment was started 5-7 weeks after skin implantation in mice with early-onset AA. Erianin was administered via intraperitoneal injection (250 μg per mouse) twice a week for a total of 8 weeks. Hair growth was documented through photography, and the incidence of AA was then calculated. Mice were sacrificed by cervical dislocation, and tissues were collected for subsequent experiments.

Enzyme-linked immunosorbent assay (ELISA)

CD8+ T cells were isolated and cultured from mouse SDLNs (spleen draining lymph nodes), and anti-CD3/CD28-stimulated T cells were used for detecting IFN-γ and IL-2 using enzyme-linked immunosorbent assay kit (ELISA, BioLegend). Briefly, cells were cultured for 24 h, and the culture medium was collected and centrifuged to obtain the supernatant. The 96-well

plates were pre-coated with anti-IL-2 or anti-IFNγ antibody. A volume of 100 µl supernatant was added into each well and incubated for 60 min at 37°C. After washing to remove unbound substances, a detection antibody was added for incubation and binding. Following additional washes, the enzyme conjugate (Streptavidin-HRP) was added for incubation and binding. Then, the substrate TMB was added to react and visualization. The reaction was terminated by adding a termination solution, and the absorbance was measured at 450 nm.

Flow cytometric analysis

Cell suspensions were prepared from skin tissue and SDLNs. The skin was pretreated by degreasing with 0.25% trypsin, separating the epidermis from the dermis, and digesting with type IV collagenase (1 mg/ml) and deoxyribonuclease I (0.05 mg/ml; Sigma-Aldrich). The digested suspension was filtered through 70-µm cell strainers and washed. While SDLN tissues were processed by grinding, filtration, and washing. The prepared cell suspensions were stained with various fluorescence-coupled antibodies and analyzed using a flow cytometer (BD Biosciences). Data on cell expression frequency were processed and analyzed using FlowJo software.

Immunofluorescence staining

Mouse skin tissues were collected, and frozen sections were prepared following acetone fixation, sucrose gradient dehydration, and OCT embedding. Subsequently, the tissues were blocked with 5% donkey serum. For staining, the sections were incubated overnight at 4°C with primary antibodies, including anti-CD8, anti-MHC I, and anti-MHC II. The next day, fluorescence-coupled secondary antibodies were applied and incubated for 2 hours at room temperature. After three washes with PBS, the nuclei were stained with DAPI. Finally, immunofluorescence images were captured using a Zeiss LSM 700 confocal microscope.

Statistics analysis

All data were statistically analyzed using GraphPad Prism 7.0 software. A two-tailed Student's t-test was used to compare two data sets. Log-rank test was used to analyze hair loss or regrowth curves. One-way analysis of variance (ANOVA) analysis was performed for comparisons among multiple groups. Data are presented as mean ± standard deviation (SD) in bar and dot plots. Statistical significance thresholds were as follows: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

Results

Erianin systemic treatment reversed AA

The effectiveness of Erianin in reversing AA was initially assessed using systemic treatment. In a 12-week trial on C3H/HeJ AA mice, those treated with Erianin exhibited a marked increase in hair regrowth compared to the control group (**Figure 1A**, **1B**). Immunofluorescence staining of the mouse skin revealed a significant reduction in inflammation-related markers in the Erianin-treated group, further supporting the observed hair regrowth (**Figure 1C**). Additionally, flow cytometric analysis demonstrated a notable decrease in the frequency of immune cell populations infiltrating the mouse skin post-treatment (**Figure 1D**, **1E**). These findings collectively indicate that Erianin effectively inhibits IFN-γ signaling and may serve as a systemic therapy for AA.

Erianin effectively inhibited the development of AA in C3H/HeJ-transplanted mice

The potential of Erianin to prevent AA was assessed through systemic treatment in C3H/HeJ-transplanted mice. After a 4-week Erianin treatment, mice were closely monitored for signs of hair loss. Remarkably, the Erianin-treated group showed no signs of hair loss throughout the 12-week observation period, while the control group began losing hair by week 5, with substantial hair loss observed by week 9 (**Figure 2A**, **2B**). Immunofluorescence staining revealed a significant reduction in the expression of inflammation-related markers CD8, MHC I, and MHC II in the skin of Erianin-treated mice (**Figure 2C**). Flow cytometric analysis further demonstrated a significant decrease in the frequency of CD8+NKG2D+ T cell and CD8+CD44+CD62L- T cell infiltration following Erianin treatment (**Figure 2D**, **2E**). Similarly, a notable reduction in CD8+CD44+CD62L- T cells was observed in SDLNs, a crucial site for the development of desmoplastic T cells. These findings highlight the potential of Erianin to effectively inhibit the proliferation of alopecia-associated T cells, thereby contribut-
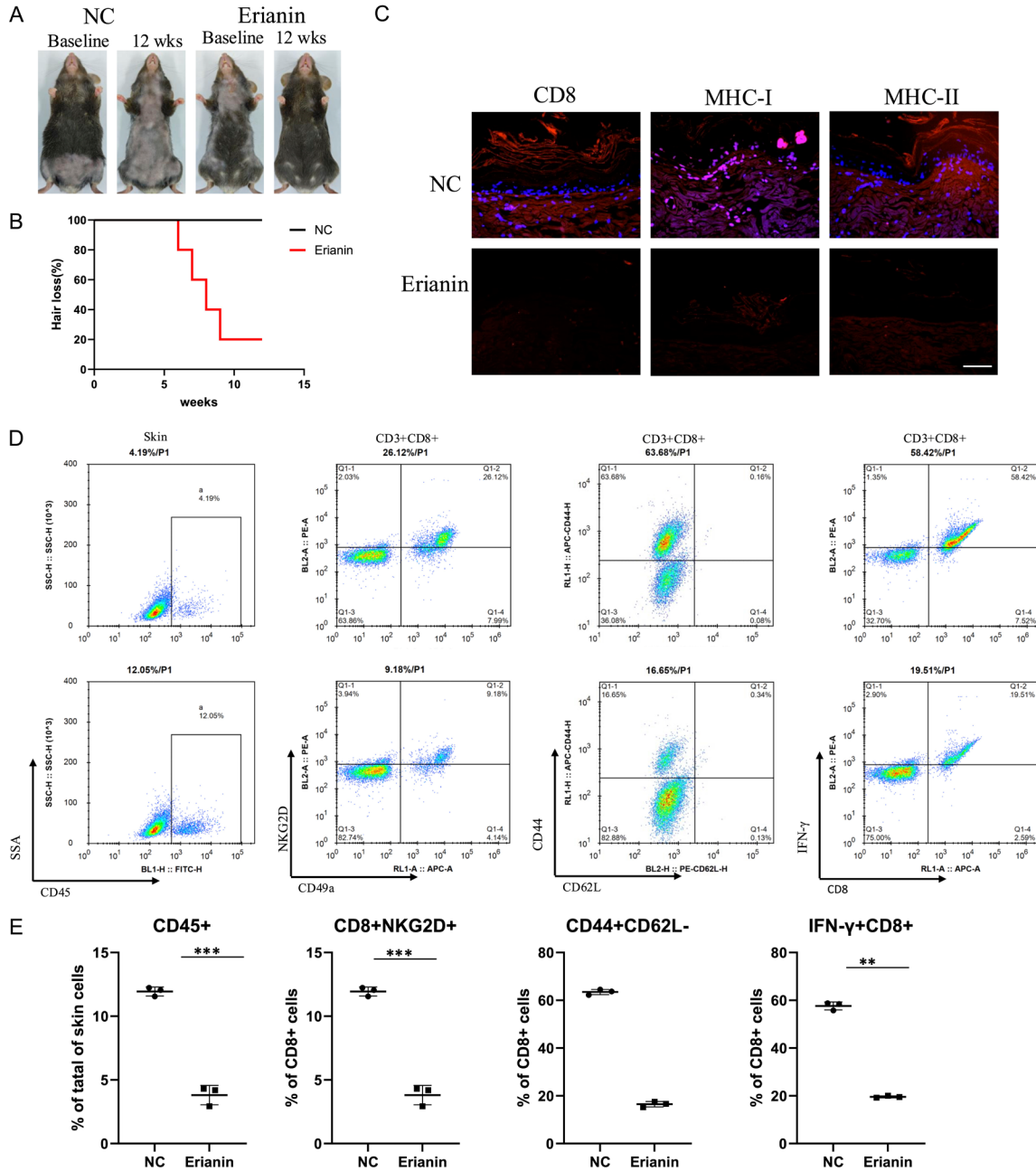
Figure 1. Erianin treatment reversed alopecia areata (AA). A. Images of C3H/HeJ mice before and after Erianin treatment. B. Statistics of hair loss over the weeks following treatment. C. Immunofluorescence images of mouse skin sections stained with anti-CD8, anti-MHC I, or anti-MHC II monoclonal antibodies. D. Infiltration of CD45+ leukocytes, CD44+CD62L-CD8+ T cells, NKG2D+CD8+ T cells, and IFN-γ+CD8+ T cells in the mouse skin after Erianin treatment detected by Flow cytometry. E. Quantitative results of 1D. Scale bar =50 μm. NC, untreated mice. **P<0.01, ***P<0.001.

ing to the successful inhibition of AA in C3H/HeJ-transplanted mice.

Topical treatment with Erianin restored hair growth in AA mice

Topical administration is a commonly used way for AA management, offering the advantage of

minimizing adverse reactions. This study assessed the effects of Erianin's topical application. Similar to the results observed with systemic administration, **Figure 3A** shows that topical Erianin treatment significantly promoted hair regrowth in AA mice. Immunofluorescence staining (**Figure 3B**) and flow cytometric analy-
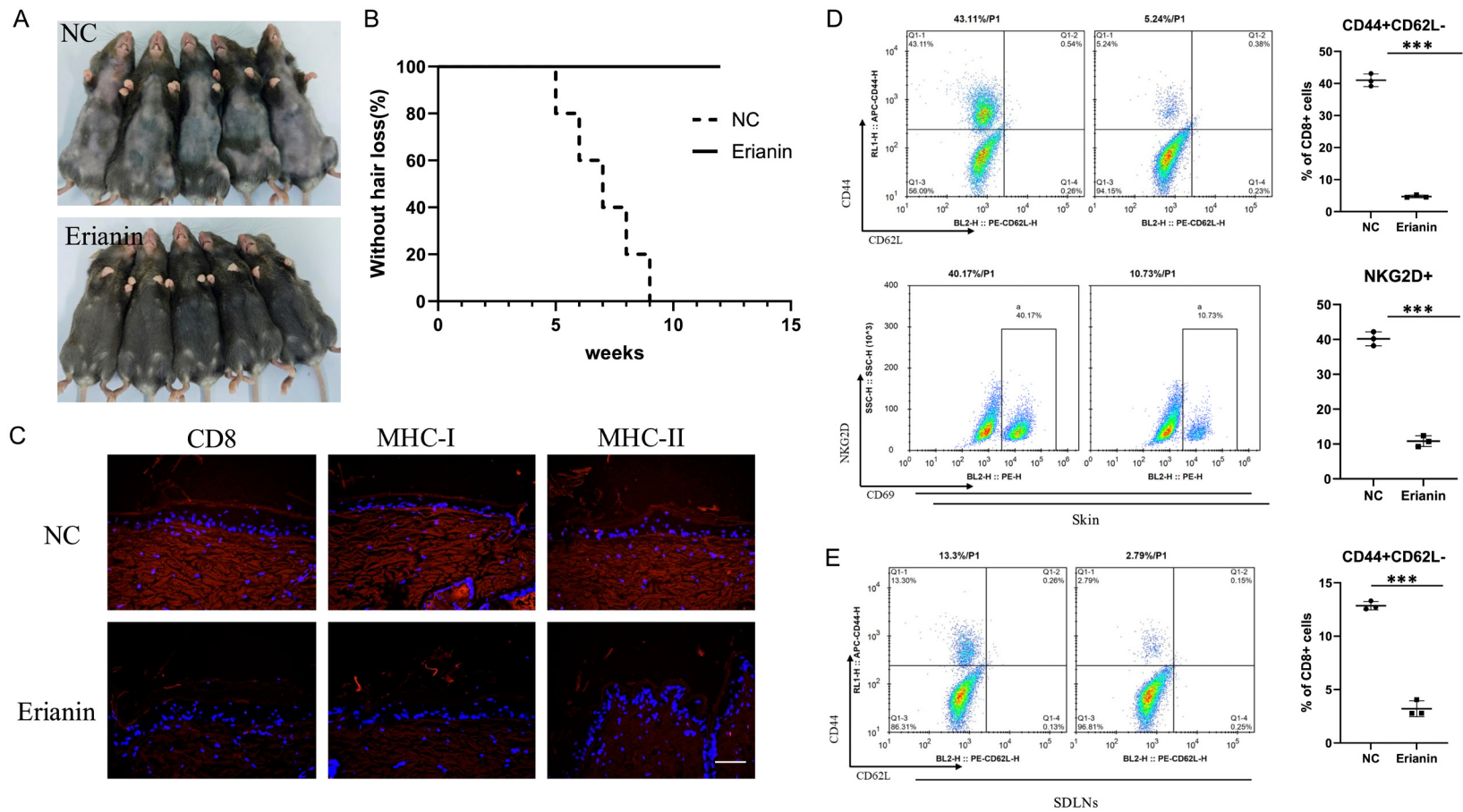
Figure 2. Erianin treatment inhibits the development of alopecia areata (AA) in C3H/HeJ-transplanted mice. A. Images of Erianin-treated and control mice. B. Statistics of hair loss in the mice of two groups. C. Immunofluorescence images of mouse skin sections stained with anti-CD8, anti-MHC I, or anti-MHC II monoclonal antibodies. D. CD44+C-D62L-CD8+ T cell and NKG2D+CD8+ T cells populations in the skin of two groups of mice detected by Flow cytometry. E. CD44+CD62L-CD8+ T cell population in skin-draining lymph nodes (SDLNs) of two groups of mice detected by Flow cytometry. Scale bar =50 μm. NC, untreated mice. ***P<0.001.
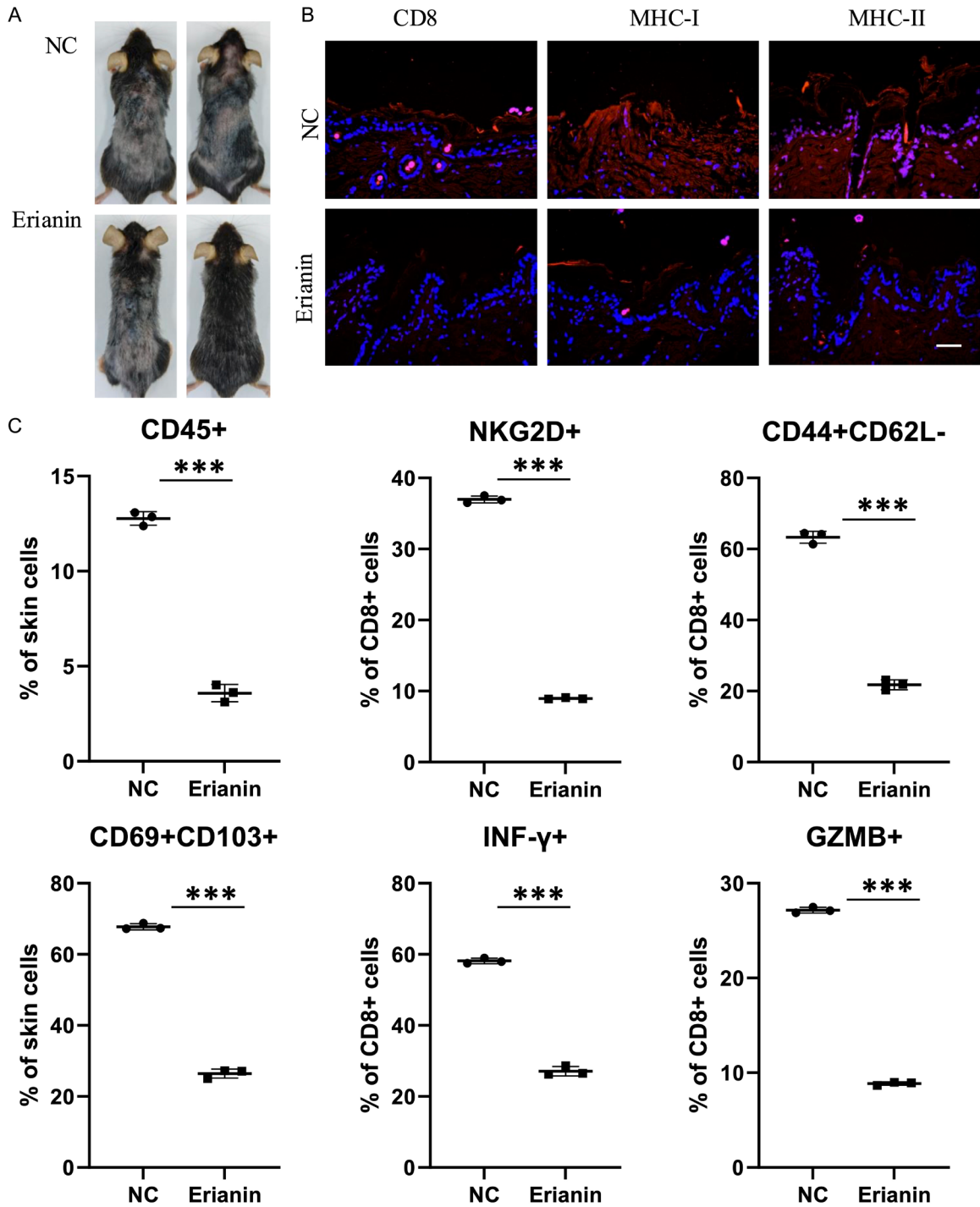
Figure 3. Erianin restored hair growth in mice with alopecia areata (AA). A. Images of hair loss or re-growth on the backs of C3H/HeJ mice before and after Erianin treatment for chronic AA. B. Immunofluorescent staining images of CD8, MHC I and MHC II were obtained from skin sections of mice before and after treatment. C. Immune cell populations (CD45+ leukocytes, NKG2D+CD8+ T cells, CD44+CD62L-CD8+ T cells, CD103+CD69+CD8+ T cells, IFN-γ-producing CD8+ T cells, and GZMB-or PRF1-producing CD8+ T cells) in the skin of mice before and after treatment. Scale bar =50 µm. NC, untreated mice. ***P<0.001 (one-way ANOVA).

sis (**Figure 3C**) revealed a noticeable reduction in inflammatory markers and immune cells in the skin of treated mice compared to the controls. These findings suggest that topical Erianin

treatment is as effective as systemic therapy and holds promise for promoting hair growth in AA mice.

Erianin inhibited the function of T effector cells and facilitated immune modulation

To investigate the immunological effects of Erianin, the study focused on its impact on various T cell populations. CD3/CD28 antibody stimulation was performed on both control and Erianin-treated mice to activate T cells. Subsequently, ELISA was performed to assess the levels of IFN-γ and IL-2 produced by CD8+ T cells from SDLNs of treated mice. As shown in **Figure 4A**, Erianin treatment significantly reduced the expression of these two cytokines. Following this, CD8+ T cells isolated from SDLNs of C3H/HeJ AA mice were used to re-stimulate the treated mice to evaluate the function of effector T cells. As anticipated, all mice in the control group developed varying degrees of AA after stimulation with the same number of SDLN CD8+ T cells, while none of the Erianin-treated groups developed AA for 15 weeks (**Figure 4B**, **4C**). Flow cytometry was subsequently used to analyze lymphocyte populations in the mouse SDLNs before and after treatment. Notably, Erianin treatment significantly reduced the content of CD19+B cells, CD3+ T cells, CD4+ T cells, and CD8+ T cells compared to the control group (**Figure 4D**). Furthermore, suppressor T cell populations in the SDLNs was also determined, revealing a significant increase in the numbers of PD-1+CD4, PD-1+CD8+ T cells (**Figure 4E**, **4F**) and FOXP3, CD4 (**Figure 4G**, **4H**) after Erianin treatment. Altogether, these findings demonstrate that Erianin treatment effectively reduces the frequency of effector T cells associated with AA while promoting immune regulation.

Discussion

The hair growth cycle is regulated by various signaling pathways, proteins and cytokines [20]. While the mechanisms by which these factors influence hair growth have been confirmed, the application of natural medicines remains limited. To address these, effort should be paid to develop natural medicines and investigate their pharmacological activities [21]. For example, the roles of numerous signaling pathways, bone bridging proteins, IGF, FGF, and other influencing factors in hair growth have not yet

been studied in the context of Chinese herbal medicines, despite their experimentally confirmed association with hair growth [22, 23]. A deeper understanding of the causes and pathophysiology of hair loss is essential for developing more effective treatments. Therefore, the exploration of natural medicines for hair growth is a promising and necessary area of research. Erianin, a small molecule bibenzyl analog derived from *Dendrobium* species of the Orchidaceae family, has been found to induce programmed cell death, inhibit angiogenesis, and exhibit antioxidant properties, with the potential to treat various diseases such as tumors, inflammation, diabetic nephropathy, retinopathy, and psoriasis [24, 25]. In this study, we examined the effects of Erianin on AA mice, aiming to evaluate its therapeutic potential. Specifically, we investigated its impact on hair regeneration and its ability to inhibit T cell-mediated hair loss, aiming to elucidate its molecular mechanisms in promoting hair growth and immune regulation.

Current domestic and international guidelines for treatment of alopecia areata (AA) consistently recommend various modalities, including general treatment, local treatment, systemic treatment, and other therapies [26]. Specifically, local immunotherapy is suggested for patients with long-standing disease and those unresponsive to other treatments. In pediatric patients with pemphigus vulgaris, pemphigus, or pemphigus oculosus, contact immunotherapy is recommended as a first-line option before considering systemic treatments [27, 28]. In our study, Erianin was administered to C3H/HeJ AA mice, demonstrating its ability to reverse the disease and restore hair growth, regardless of whether it was used systemically or topically. Molecular analysis revealed that Erianin participates in various immune processes related to AA progression. It effectively inhibited the secretion of pro-inflammatory cytokines by skin monocytes, reduced CD8+ T cell infiltration and expression, and played a vital role in regulating the proliferation and activation of desmoplastic T cells in SDLNs. Notably, memory T cells in peripheral tissues, which contribute to immunosuppression upon re-invading pathogens, were also influenced by Erianin. Through modulation of the IFN-γ signaling pathway, Erianin exerted suppressive effects on cell populations associated with AA,
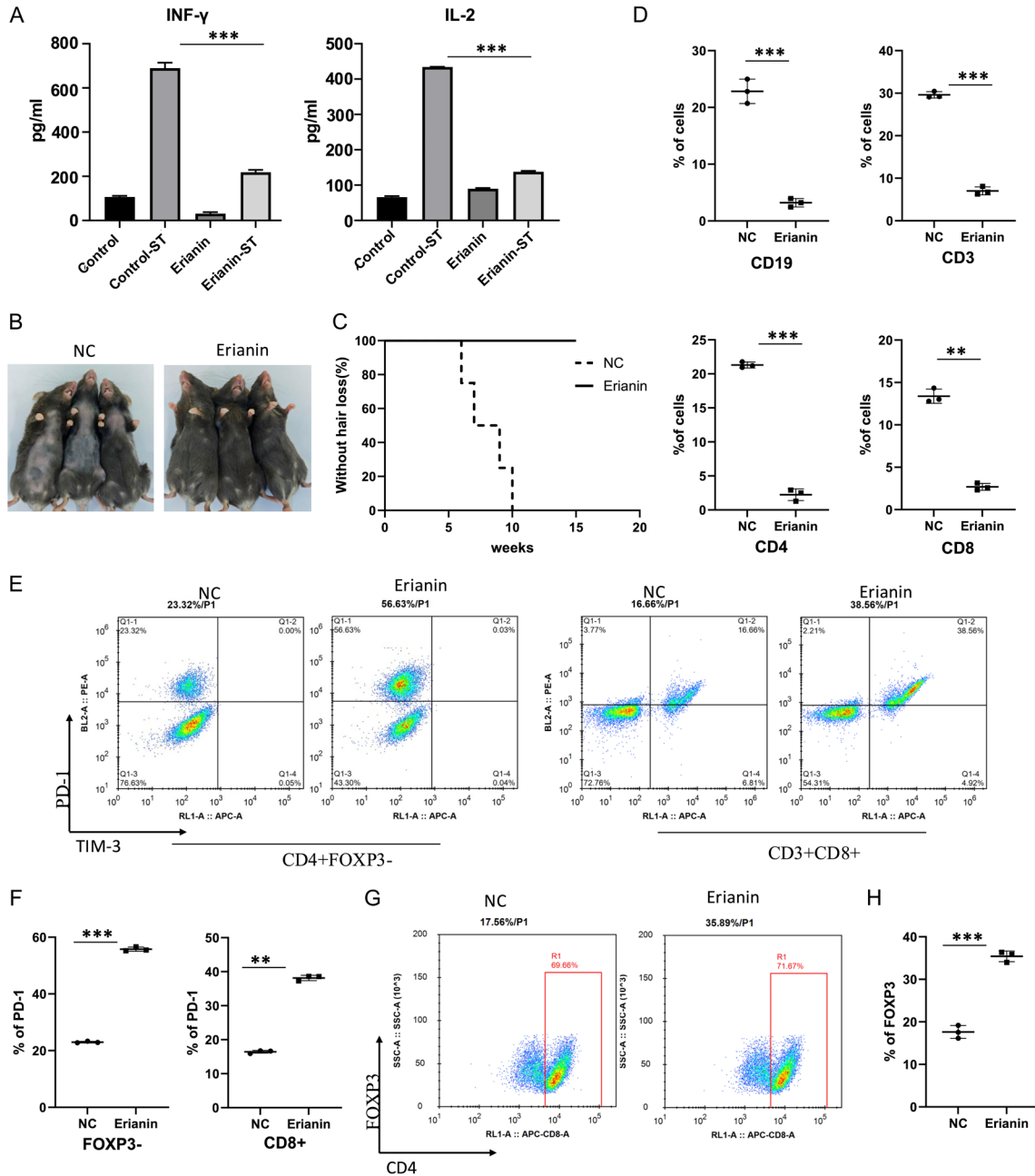
Figure 4. Erianin suppressed effector T cells. A. Changes in cytokines (IFN-γ, IL-2) produced by T cells in the skin-draining lymph nodes (SDLNs) of control and Erianin-treated mice after 48 hours of stimulation with anti-CD3/CD28. B. Pictures of hair growth in mice. C. Incidence of AA after 10 weeks of treatment with CD8+ T cells isolated from the SDLNs of C3H/HeJ AA mice in the control and Erianin-treated groups. D. Proportion of immune cell subsets (CD19, CD3, CD4, and CD8) in the SDLNs of C3H/HeJ AA mice before and after treatment with Erianin. E, F. Proportion of PD-1+ T cells and Treg cells before and after Erianin treatment detected by Flow cytometry. G, H. Proportion of FOXP3 and CD4 before and after Erianin treatment detected by Flow cytometry. NC, untreated mice. **P<0.01, ***P<0.001 (one-way ANOVA).

including IFN-γ+-producing CD8+ T cells [29]. These findings underscore Erianin's robust inhibitory effects and its potential as an effective treatment for AA.

Conclusion

In conclusion, this study highlights the significant therapeutic role of Erianin in treating alo-

pecia areata. Both topical and systemic administration of Erianin demonstrated effective prevention and reversal on AA, underscoring its potential as a versatile and impactful treatment option.

Disclosure of conflict of interest

None.

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