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Modulating immune responses in alopecia: therapeutic insights and potential targets of antisense oligonucleotides

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Abstract

Background Alopecia areata (AA) are hair loss disorders with distinct pathogenetic mechanisms involving immune dysregulation and microRNA modulation. AA, a T cell-mediated autoimmune disease, is characterized by sudden hair loss, with interferon-gamma (IFN-γ) playing a pivotal role in pathogenesis. The upregulation of IFN response genes, including IFN-inducible chemokines CXCL9, CXCL10, and CXCL11, in lesional skin reflects the activation of the IFN response pathway and contributes to immune cell recruitment and inflammation.

Results Recent research highlights the role of SIRT1, a class III histone deacetylase, in modulating immune responses in AA. SIRT1 inhibition promotes the production of Th1 cytokines and chemokines, impairing inflammation, while SIRT1 activation suppresses autoreactive responses through NF-κB deacetylation and STAT3 phosphorylation. Additionally, antisense oligonucleotides (ASOs) targeting miR-485-3p show therapeutic potential in promoting hair regrowth and mitigating inflammation in murine models of androgenic alopecia (AGA) and AA.

Conclusion Understanding chemokine dysregulation provides key insights into AA pathogenesis and highlights TAMI-M as a potential therapy for reducing inflammation and promoting hair regeneration. These findings advance the exploration of immune, microRNA, and SIRT1 pathways as targets for novel hair loss treatments.

Keywords Alopecia areata, C3H/HeJ mice, Antisense oligonucleotides, SIRT1, Outer root sheath

Introduction

Alopecia areata (AA) and androgenic alopecia (AGA) are distinct hair loss disorders characterized by different pathogenetic mechanisms. AA is an autoimmune condition mediated by T cells, resulting in sudden and often patchy hair loss, while AGA is driven by androgens and typically manifests as a progressive thinning of hair in a characteristic pattern [1–7]. Despite their differing etiologies, recent research has highlighted common underlying molecular pathways, particularly involving the dysregulation of microRNAs (miRNAs) and key cellular signaling molecules [8–12].

One such molecule of interest is SIRT1, a member of the sirtuin family of NAD+dependent protein

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deacetylases, has emerged as a critical regulator of various cellular processes, including inflammation, oxidative stress, and apoptosis [13, 14]. Studies have shown dysregulation of SIRT1 expression in both AA and AGA, suggesting its involvement in the pathogenesis of these conditions [13, 15, 16]. Interestingly, a recent report implicated miR-485-3p, a regulator of SIRT1 mRNA, in the inflammatory response associated with Alzheimer's disease [17]. SIRT1 plays a pivotal role in promoting hair follicle health by protecting hair follicle stem cells (HFSCs) from TNFα-mediated inflammatory stress, maintaining mitochondrial integrity, and modulating immune responses [13, 16, 18]. Its ability to suppress pro-inflammatory cytokines and preserve the immune privilege of hair follicles highlights its therapeutic potential in autoimmune hair loss disorders such as alopecia areata.

In AA, aberrant immune responses lead to the upregulation of pro-inflammatory cytokines and chemokines, including NF-κB and its downstream targets CXCL9, CXCL10, and CXCL11 [16, 19]. These chemokines play pivotal roles in immune cell recruitment and inflammation within the hair follicles, contributing to hair loss in AA [3, 16, 20, 21]. Conversely, in AGA, androgens such as dihydrotestosterone (DHT) exert their effects through the androgen receptor (AR) pathway, leading to follicular miniaturization and hair thinning.

Recent studies have implicated miRNAs as key regulators of gene expression in both AA and AGA [22, 23]. Of particular interest are miRNAs targeting SIRT1, which have been shown to modulate SIRT1 expression levels and activity. By targeting SIRT1, these miRNAs may disrupt cellular homeostasis and contribute to the pathogenesis of AA and AGA.

In this context, targeting SIRT1 with ASOs directed against specific miRNAs presents a promising therapeutic approach for both AA and AGA. By restoring SIRT1 activity, ASOs may mitigate inflammation, oxidative stress, apoptosis and immune responses in the hair follicles, thereby promoting hair regrowth and halting the progression of hair loss in affected individuals.

This study aims to explore the interplay between SIRT1, NF-κB, and chemokines in the pathogenesis of AA and AGA. By elucidating the shared molecular mechanisms underlying these conditions, we seek to identify novel therapeutic targets for treating hair loss. Specifically, we conducted in vivo experiments to investigate the effects of TAMI-M on modulating SIRT1 activity and NF-κB signaling in relation to hair growth and inflammation in animal models of AA and AGA. Additionally, we analyzed the expression levels of CXCL9, CXCL10, and CXCL11 in murine hair follicles and the immune response in human outer root sheath cells (HORS) to assess their correlation with disease severity.

Through controlled treatments, including androgen therapies and immunomodulatory agents, this study aims to provide direct evidence of SIRT1's immunoregulatory role in maintaining hair follicle homeostasis. By utilizing advanced experimental approaches, we aim to identify critical molecular targets that link AA and AGA, paving the way for the development of effective therapeutic strategies for these complex hair loss disorders.

Materials and methods

Animals

C3H/HeJ mice (age: 15 weeks old) were spontaneously developed AA obtained from Central Lab, Animal Inc, Seoul, Republic of Korea. A total of 20 female mice were observed for development of alopecia and housed under standard conditions (12 h light/dark cycle, 22–24 °C) with ad libitum access to standard chow diet and water. The animal experimentation conducted in this study adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of BIORCHESTRA (permit numbers: BOIA-CUC-20230531-0002). The mice were randomly allocated into four groups, with five mice in each group, to ensure consistent experimental conditions.

Alopecia model induction

Mice were injected subcutaneously with polyinosinic: polycytidylic acid [Poly (I: C)] (100 μg/mice) twice a week (frequency) and IFNγ (2×10^4 units/mice) once a week for alopecia induction and further promote alopecia development [16, 21].

Treatment with TAMI-M

Following alopecia induction, mice were randomly assigned two treatment groups ($n = 5$ mice/group): a weekly treatment group and a monthly treatment group (Fig. 1A). Both groups received treatments formulated with TAMI-M dissolved in RNase-Free Water. Mice in the weekly treatment group were administered subcutaneous injections of TAMI-M at a dose of 0.8 mg/kg (mpk) body weight once per week. In contrast, the monthly treatment group received the same dose via subcutaneous injection once per month throughout the 12-week study duration. The aim of the experiment was to evaluate the protective effect of TAMI-M against the induction of alopecia areata (AA) based on the injection frequency (weekly versus monthly).

Assay for in vivo hair growth in C57BL/6J mice

The animal experimentation conducted in this study adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee

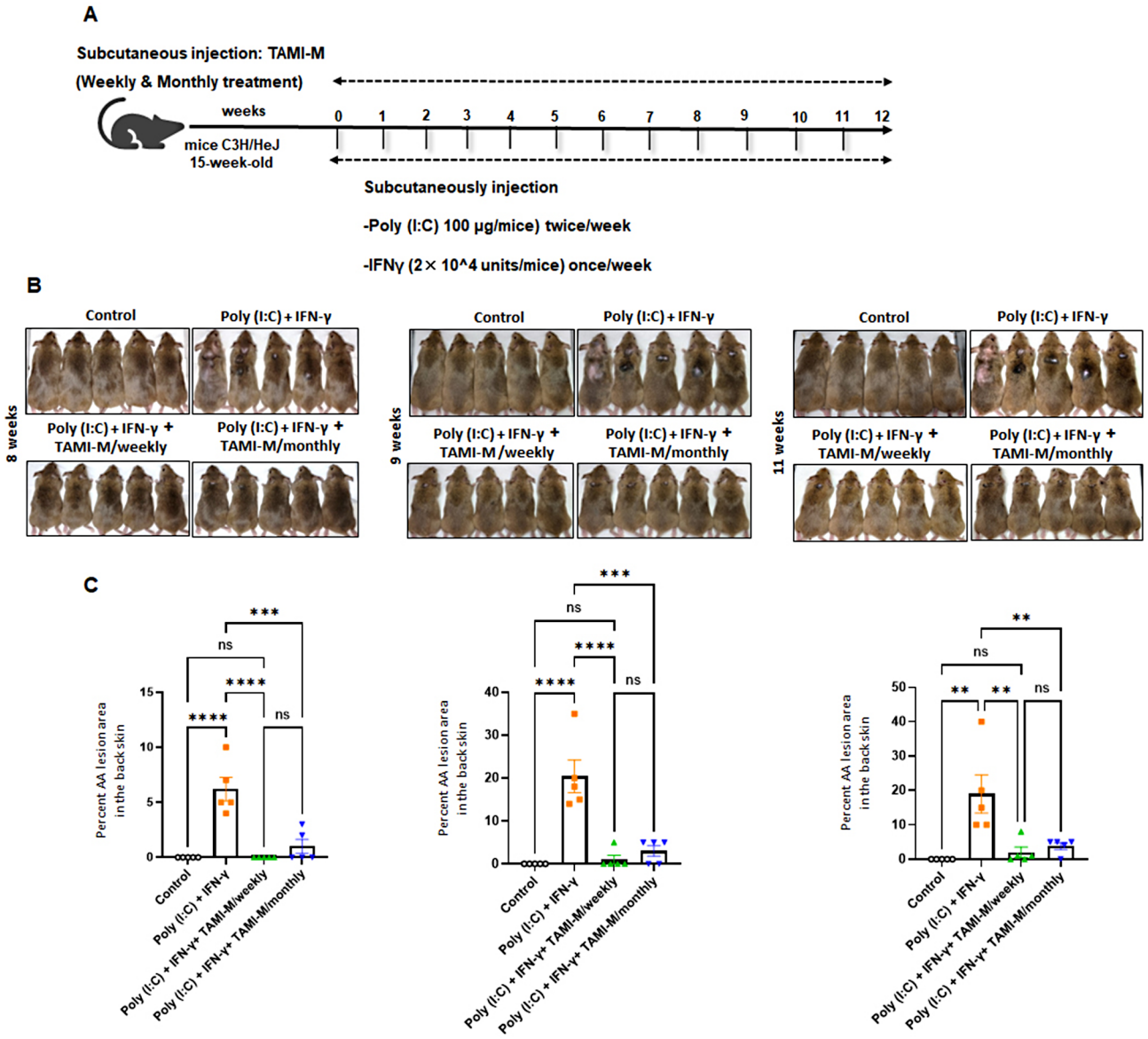

Fig. 1 Efficacy of TAMI-M weekly and monthly treatments in C3H/HeJ AA model Mice: **A** Schematic representation of the experimental design. Fifteen-week-old C3H/HeJ mice were injected subcutaneously with poly I: C and IFN-γ to induce AA-like symptoms. TAMI-M was administered subcutaneously at a dose of 0.8 mg/kg either weekly or monthly, while control animals received PBS (vehicle). Each treatment group consisted of 5 mice ($n = 5$). **B** Representative images of hair regrowth and alopecia severity were taken at defined intervals post-treatment on 8, 9 and 11 weeks. Images show the extent of lesional area before and after treatment in the TAMI-M weekly, monthly, and vehicle groups. **C** Quantitative analysis of AA lesion areas in the dorsal skin of treated and control mice was performed. Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). The effects of TAMI-M treatment against AA lesion formation were significant for both dosing regimens in the presence of poly I: C and IFN-γ treatment. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle (before poly I: C induction); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle (after poly I: C induction)

(IACUC) of BIORCHESTRA (permit number: BOIA-CUC-20220908-0001. Six-week-old, specific-pathogen-free C57BL/6J mice were obtained from DBL Co. Ltd. and housed in cages under controlled conditions (temperature: 23 °C ± 3 °C, humidity: 40–70%). Animal studies were conducted in accordance with ethical guidelines to ensure the well-being of the animals, with efforts to minimize suffering and distress. Inhalation anesthesia (Isoflurane 3–5% with oxygen) was administered during hair growth evaluations. In vivo studies were performed to assess the efficacy of TAMI-M, delivered via subcutaneous injection, in a DHT-induced androgenetic alopecia (AGA) mouse model. Animals were monitored every other day for health and behavior, and no deaths or adverse effects were observed. All animals completed the

study protocol as planned, with no instances of euthanasia or mortality.

A humanitarian endpoint scoring system was employed based on the research purpose of the hair growth study. This system included criteria such as weight change, hair condition, eyes, and nose health, as well as movement and posture, with a score of 0 indicating no abnormalities in these parameters. Previous oligonucleotide studies revealed no significant differences between vehicle control and treatment with scramble control (negative control, #AM17010). miR-485-3p ASO (AGAGAGGAGAG CCGUGUAUGAC) was synthesized by Integrated DNA Technologies (Coralville, IA, USA) [17]. The non-targeting ASO (negative control, #AM17010) was purchased from Thermo Fisher (Waltham, MA, USA). Dosage optimization for TAMI-M was performed prior to the current study. A range of doses (0.1–0.8 mg/kg data not shown) was tested in preliminary in vivo studies to evaluate efficacy and safety. The selected dose of 0.8 mg/kg demonstrated maximal therapeutic benefit with no observed adverse effects, supporting its use in the current experiments.

Therefore, ASO was administered twice via intramuscular (IM) injection (0.3 and 0.6 mg/kg/50µl) on Days 0 and 7. Animals received DHT subcutaneously injected 1 d before depilation and thereafter every other day (1 mg/100 µL) to delay in hair regrowth and induced hair miniaturization in C57BL/6 mice. The euthanasia method employed for animals included CO_2 gas inhalation as well as cervical dislocation. To test hair regrowth efficacy, mouse hair was shaved using a small animal clipper and they were subsequently depilated with hair removal cream one day before (Day 0) ASO administration. Skin pigmentation and hair growth were observed and recorded using digital photomicrographs. Each panel of mice, typically consisting of multiple (3 to 6) animals, photographed together at the same time for a specific time point standardizing lighting conditions, background, and positioning of the animals within the frame. An index of hair growth visual scoring guide in C57BL/6 mice was used, with scores ranging from 0 to 6. The pigmentation level and density of hair shaft were the factors used to determine the values, where a value of 0 indicated the absence of hair growth and pigmentation, and higher values were indicative of greater hair growth density and darker skin. Images shown are representative of a 0-to-6 hair regrowth quantification scale, where grade 0 indicates pink skin, no pigmentation; grade 1, < 30% skin area showing darkening with no visible hair; grade 2 = 30–70% skin area showing darkening with no visible hair; grade 3 = > 70% skin area showing darkening with visible hair; grade 4 = > 70% skin area showing darkening and 30–70% visible hair; grade 5 = > 70% skin area showing darkening and > 70% visible hair; and grade 6 = > 90% skin area showing darkening and > 90% visible hair.

Human outer root sheath cell (ORS)

Human ORS cells were obtained from the ScienCell Research Laboratory (HHORSC: Catalog #2420). ORSC were cultured in Mesenchymal Stem Cell Medium (MSCM, Cat. #7501) supplemented with 10% FBS exosome free and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Subculture when the culture reaches 90–95% confluency. Rinse the cells with DPBS and incubated with 0.25% trypsin and 0.02% ethylenedia-minetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Hair follicles were vigorously pipetted to obtain single-cell populations. The dissociated cells were rinsed in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), centrifuged 1000 rpm for 5 min. Gently resuspend cells in culture medium count and transfer the suspended cells to the cell culture plate.

Reagents

Poly(I: C) was obtained from InvivoGen (San Diego, CA, USA). Recombinant murine IFN-γ was sourced from Peprotech (Catalog #315-05, Rocky Hill, NJ, USA). Ruxolitinib (phosphate, CAS#: 1092939-17-7) was acquired from MedKoo Biosciences, USA.

Quantitative RT-PCR

Total RNA was isolated using the small and large RNA kit (Macherey-Nagel, Allentown, PA, USA) and QIAzol lysis reagent (Qiagen, Hilden, Germany). miScript II RT Kit (Qiagen) was used for cDNA synthesis for miRNA detection and quantification. The reverse-transcribed product was subjected to qRT-PCR using TaqMan™ miRNA assay (ThermoFisher Scientific, Waltham, MA, USA) and primers were used: miR-485-3p forward: 5'-GTCATACACGG CTCTCCTCTCTAA-3', reverse: 5′- GAATCGAGCACC AGTTACG-3′, probe: FAM-CGAGGTCGACTTCCTAG A-NFQ. RNU6 forward: 5'-GCTTCGGCAGCACATATA CTAAAAT-3, reverse: 5′- GAATCGAGCACCAGTTAC G-3′, probe: FAM-CGAGGTCGACTTCCTAGA-NFQ. Relative gene expression was analyzed using the 2−ΔΔct method. TOPscript™ RT DryMIX (Enzynomics, Daejeon, Korea) was used for cDNA synthesis for mRNA detection and quantification. The reverse-transcribed product was subjected to qRT-PCR using TOPrealTM qPCR 2 × PreMIX, SYBR Green (Enzynomics), and primers. PCR primers were commercially synthesized (Bioneer, Daejeon, Korea). A 40-cycle amplification was applied for all primers using the CFX96 Real-Time System (Bio-Rad). The primer sequences used for mRNA expression analysis are listed in Supplementary Table 1.

Western blot analysis

Cells were lysed in Proprep solution (Intron, Deajeon, Korea). Total protein was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Samples were run on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with appropriate primary antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Intron). following primary antibodies: rabbit anti-IL-6 (Cat# ab208113, RRID: AB_2927421, Abcam; 1:1000), mouse anti-TNF-α (Cat# sc-52746, RRID: AB_630341, Santa Cruz Biotechnology; 1:1500), mouse anti-NLRP3 (Cat# AG-20B-0014-C100, RRID: AB_2885199, AdipoGen; 1:1500), rabbit anti-AR (Cat# D6F11, RRID: AB_2799166, Cell Signalling Technology; 1:2000), Mouse monoclonal anti-SIRT1 antibody (Cat#ab110304, RRID: AB_10864359, abcam; 1:1000), rabbit anti-CD36 (Cat# PA1-16813, RRID: AB_568487, Thermo Fisher Scientific; 1:1000), rabbit P-Stat3 (Cat# Tyr705, RRID: AB_2716460, Cell Signalling Technology; 1:1000), rabbit anti-HSP70 (Cat# 4872, RRID: AB_2279841, Cell Signalling Technology; 1:1500), rabbit mAb P-NF-κB (Cat# #3033, RRID: AB_331284, Cell Signalling Technology; 1:1000). Membranes were blotted with HRP-conjugated anti-mouse (Cat# 7076, RRID: AB_330924, Cell Signalling Technology; 1:3000) or anti-rabbit (Cat# 7074, RRID: AB_2099233, Cell Signaling Technology; 1:3000) IgG secondary antibodies. Membranes were incubated overnight at 4 °C with monoclonal antibodies against β-actin (Cat# 4967, RRID: AB 330288, Cell Signalling Technology; 1:4000, Cat# sc-47778 HRP; RRID: AB_2714189, Santa Cruz Biotechnology; 1:4000). The membranes were incubated with horseradish peroxidase (HRP) conjugated with anti-IgG secondary antibodies for 2 h. ECL Western blotting detection reagent was used for exposure and development with chemiluminescence Western Blotting Detection System (ImageQuant LAS 500). Bands were quantified by densitometry using Image (version 1.53 V, NIH, Bethesda, MD, USA).

Statistical analysis

Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). One-way analysis of variance (ANOVA) was used to compare statistical significance within and among three or more groups using Prism software V10.2.3 (GraphPad Prism, RRID: SCR_002798, CA, USA). A value of $p < 0.05$ was considered to represent a statistically significant difference.

Results

Therapeutic potential of TAMI-M in the C3H/HeJ mouse model of AA

Using the C3H/HeJ mouse model of AA, we investigated the efficacy of TAMI-M in reducing hair loss and reversing alopecia-associated lesions. AA was induced using poly I: C and IFN-γ, agents that mimic the immune-mediated inflammatory processes underlying AA pathogenesis as shown in Fig. 1A.

The experimental design included two treatment regimens to assess the efficacy of TAMI-M: weekly and monthly subcutaneous injections (Fig. 1A). These regimens were chosen to investigate the impact of treatment frequency on the therapeutic outcomes and to determine whether less frequent dosing could achieve comparable efficacy. The disease progression and therapeutic responses were evaluated at 8, 9, and 11 weeks after induction, enabling a sequential analysis of the treatment effects.

In untreated mice, poly I: C and IFN-γ induction led to significant hair loss, confirming the successful establishment of the AA phenotype (Fig. 1B). In contrast, mice treated with TAMI-M displayed remarkable therapeutic efficacy. Both the weekly and monthly treatment groups demonstrated substantial reversal of hair loss, as confirmed by quantitative analysis showing a significant reduction in AA lesion areas in the dorsal skin of treated mice compared to AA controls induced with poly I: C and IFN-γ (Fig. 1B and C).

The protective effects of TAMI-M were further validated through graphical presentations, which illustrated the treatment-induced improvements reversal of lesion area over time at 8, 9, and 11 weeks (Fig. 1C).

TAMI-M suppresses proinflammatory cytokines and chemokines in C3H/HeJ mouse model of AA

To investigate the immunomodulatory effects of TAMI-M in AA, we focused on its impact on key proinflammatory cytokines and chemokines, specifically CXCL-9, CXCL-10, CXCL-11, and IFN-γR due to their role in the inflammatory cascade underlying AA pathology. The C3H/HeJ mouse model, treated with Poly I: C and IFN-γ, mimics the immune-mediated hair loss observed in human AA (Fig. 2A).

Two dosing regimens of TAMI-M were employed weekly and monthly to evaluate the influence of administration frequency on the reduction of proinflammatory signaling. Quantitative analysis revealed that TAMI-M treatment significantly reduced the expression levels of CXCL-9, CXCL-10, CXCL-11, and IFN-γR in the affected skin of experimental mice compared to AA (poly I: C and IFN-γ) controls (Fig. 2B). Weekly TAMI-M administration yielded a greater reduction in these proinflammatory markers compared to monthly treatment, highlighting

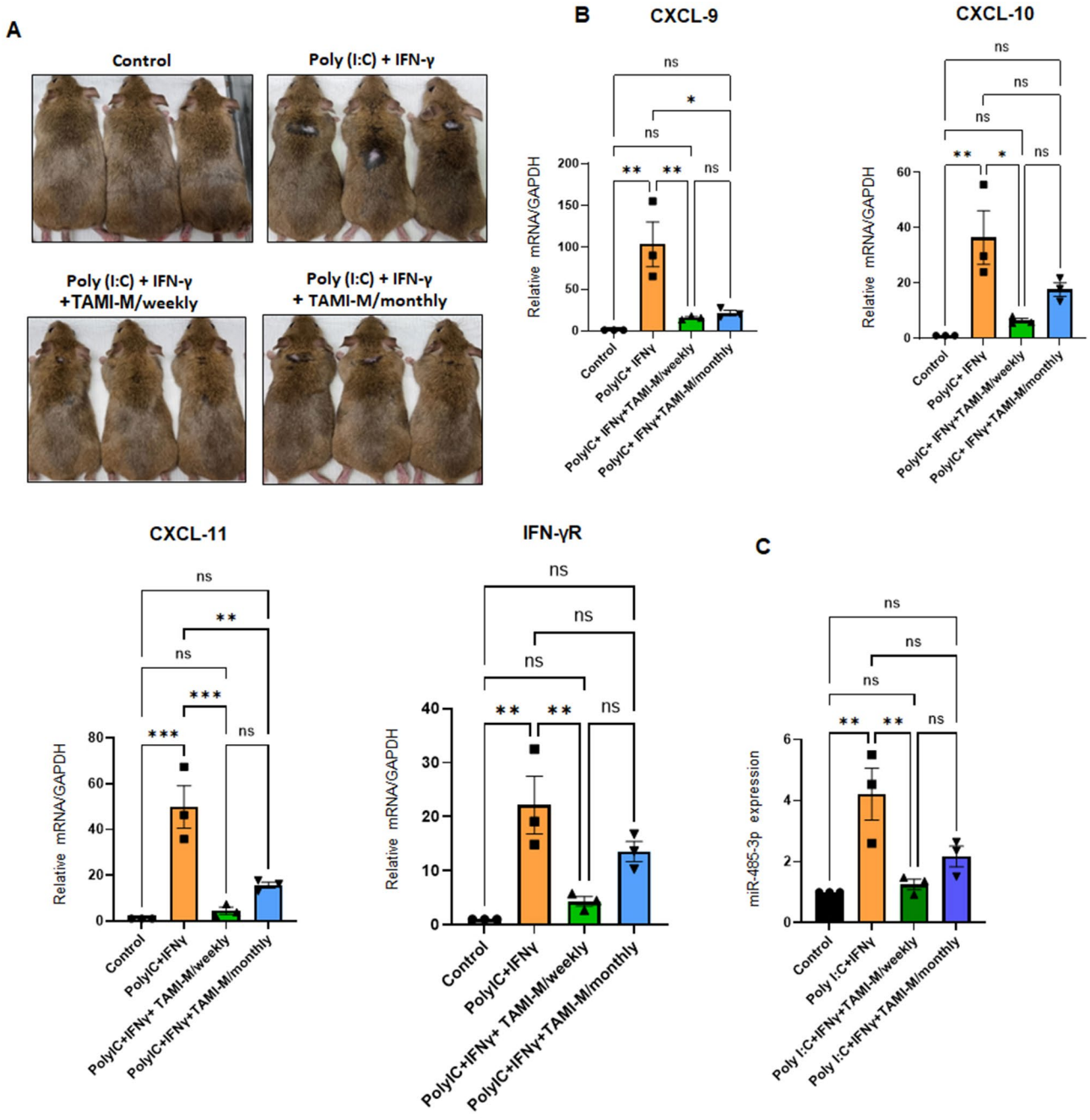

Fig. 2 Effect of TAMI-M treatment on proinflammatory chemokines, and cytokines in C3H/HeJ AA model mice: **A** C3H/HeJ mice were induced with AA-like symptoms using poly I: C and IFN-γ, mimicking the inflammatory milieu observed in human alopecia areata. TAMI-M was administered subcutaneously at a dose of 0.8 mg/kg weekly or monthly. **B** Relative expression levels of proinflammatory chemokines and cytokines were measured in dorsal skin samples via qPCR following TAMI-M treatment. Data reflect significant reductions in inflammatory markers in treated groups compared to vehicle controls. **C** miR-485-3p expression levels were evaluated in the skin of treated and control mice. Weekly and monthly TAMI-M dosing regimens both resulted in substantial downregulation of miR-485-3p expression compared to vehicle controls. Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle (before poly I: C induction); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle (after poly I: C induction)

the importance of dosing frequency in achieving optimal therapeutic outcomes.

In addition to cytokine and chemokine expression, we assessed the impact of TAMI-M treatment on miRNA-485-3p, a key regulator of SIRT1, inflammation, and hair follicle homeostasis. Weekly administration of TAMI-M resulted in a downregulation of miRNA-485-3p compared to monthly treatment (Fig. 2C).

Immunomodulatory effects of TAMI-M on proinflammatory chemokines in human ORS cells: comparison with ruxolitinib

To further elucidate the immunomodulatory effects of TAMI-M and its role in maintaining hair follicle immune privilege, we investigated its impact on the production of inflammatory mediators in human outer root sheath (ORS) cells. The ORS cells, which are follicular epidermal keratinocytes, play a crucial role as immunocompetent cells that represent a relevant model for studying the immune mechanisms underlying AA. Specifically, the regulation of proinflammatory chemokines (CXCL9, CXCL10, CXCL11, and MICA) and immune-related markers following treatment with TAMI- M.

ORS cells were treated with either TAMI-M (100 nM) or poly(I: C) (100 µg/ml) for 3 h (Fig. 3) and poly(I: C) (10 µg/ml) for 24 h (Fig. 4). Poly(I: C), a synthetic analog of double-stranded RNA, was used to simulate viral infection and trigger a proinflammatory response, thereby mimicking the immune-mediated inflammatory conditions observed in AA. This approach allowed us to evaluate whether TAMI-M could counteract these inflammatory processes in vitro.

Stimulation with poly(I: C) led to a significant upregulation of proinflammatory chemokines (CXCL9, CXCL10, CXCL11, and MICA), confirming the activation of inflammatory response (Fig. 3A). Importantly, TAMI-M significantly upregulated SIRT1, a target of miR-485-3p, which in turn downregulated the expression of NKG2D ligand MICA (Fig. 3A, B). Additionally, we assessed the expression of caspase-1, a critical component of the inflammasome a marker that significantly elevated caspase-1 levels in ORS cells, indicative of an inflammatory response and cellular damage (Fig. 3C). However, TAMI-M treatment substantially reduced caspase-1 expression, further supporting its role in mitigating inflammation and promoting hair follicle health (Fig. 3C).

Treatment with the TAMI-M significantly attenuated the Poly I: C-induced upregulation of proinflammatory chemokines, including CXCL9, CXCL10, and CXCL11 in ORS cells as well as, the Poly I: C + TAMI-M group exhibited significantly lower levels of all three chemokines (Fig. 4A).

The study employed a comparison of TAMI-M with Ruxolitinib, a clinically relevant JAK inhibitor, to benchmark the efficacy of TAMI-M against an established therapeutic agent. In parallel, ORS cells treated with Ruxolitinib exhibited a similar downregulation of CXCL9, CXCL10, and CXCL11 (Fig. 4B).

TAMI-M modulates NF-κB signaling and enhances SIRT1 expression in the AGA model

To investigate the therapeutic effects of TAMI-M in the context of androgenetic alopecia (AGA), we focused on its impact on NF-κB signaling, a key molecule involved in inflammation activation. NF-κB is a crucial transcription factor that regulates the expression of proinflammatory genes, contributing to the pathogenesis of AGA. SIRT1, a target of miR-485-3p, is known to inhibit NF-κB signaling, suggesting that miR-485-3p may modulate this pathway to reduce inflammation in the AGA model.

Western blot analysis of tissue lysates from the AGA model revealed that DHT treatment significantly elevated the phosphorylation of NF-κB (p-NF-κB), a marker of NF-κB activation. However, TAMI-M treatment effectively downregulated p-NF-κB expression, indicating that TAMI-M can counteract NF-κB activation (Fig. 5B). In addition, TAMI-M treatment resulted in a notable increase in SIRT1 expression (Fig. 5A, B), further supporting the idea that TAMI-M modulates NF-κB signaling through SIRT1.

Modulation of STAT3/IL-6 signaling by TAMI-M in AGA model

To investigate the therapeutic potential of TAMI-M in AGA, we focused on its impact on the STAT3/IL-6 signaling pathway, which plays a crucial role in inflammation and hair follicle regulation. The STAT3/IL-6 axis is known to be activated in AGA, contributing to the inflammatory environment that drives hair loss. STAT3, upon phosphorylation at tyrosine 705 (Y705), activates downstream targets that promote inflammation and AR expression, further impairing AGA. Thus, targeting this pathway could provide a therapeutic approach for mitigating the inflammatory processes underlying AGA.

Western blot analysis of tissue lysates from the AGA model revealed that DHT treatment significantly increased the phosphorylation of STAT3 (p-STAT3) and AR expression, both of which are associated with the inflammatory and androgen-driven aspects of AGA. However, treatment with TAMI-M effectively reduced p-STAT3 levels at Y705 and significantly downregulated AR expression (Fig. 5B), indicating that TAMI-M can inhibit STAT3 activation and its associated effects.

Additionally, TAMI-M treatment led to a marked reduction in IL-6 expression, further supporting the role of miR-485-3p in modulating inflammation and downstream cytokine signaling (Fig. 5B).

Therapeutic modulation of inflammatory pathways and HSP70 expression by TAMI-M in an AGA model

To investigate the therapeutic effects of TAMI-M in AGA, we focused on its potential to modulate inflammatory processes and its impact on HSP70 expression, which is involved in immune regulation and cellular stress responses. AGA is associated with progressive hair loss due to genetic and hormonal factors, and it is known to be accompanied by increased pro-inflammatory

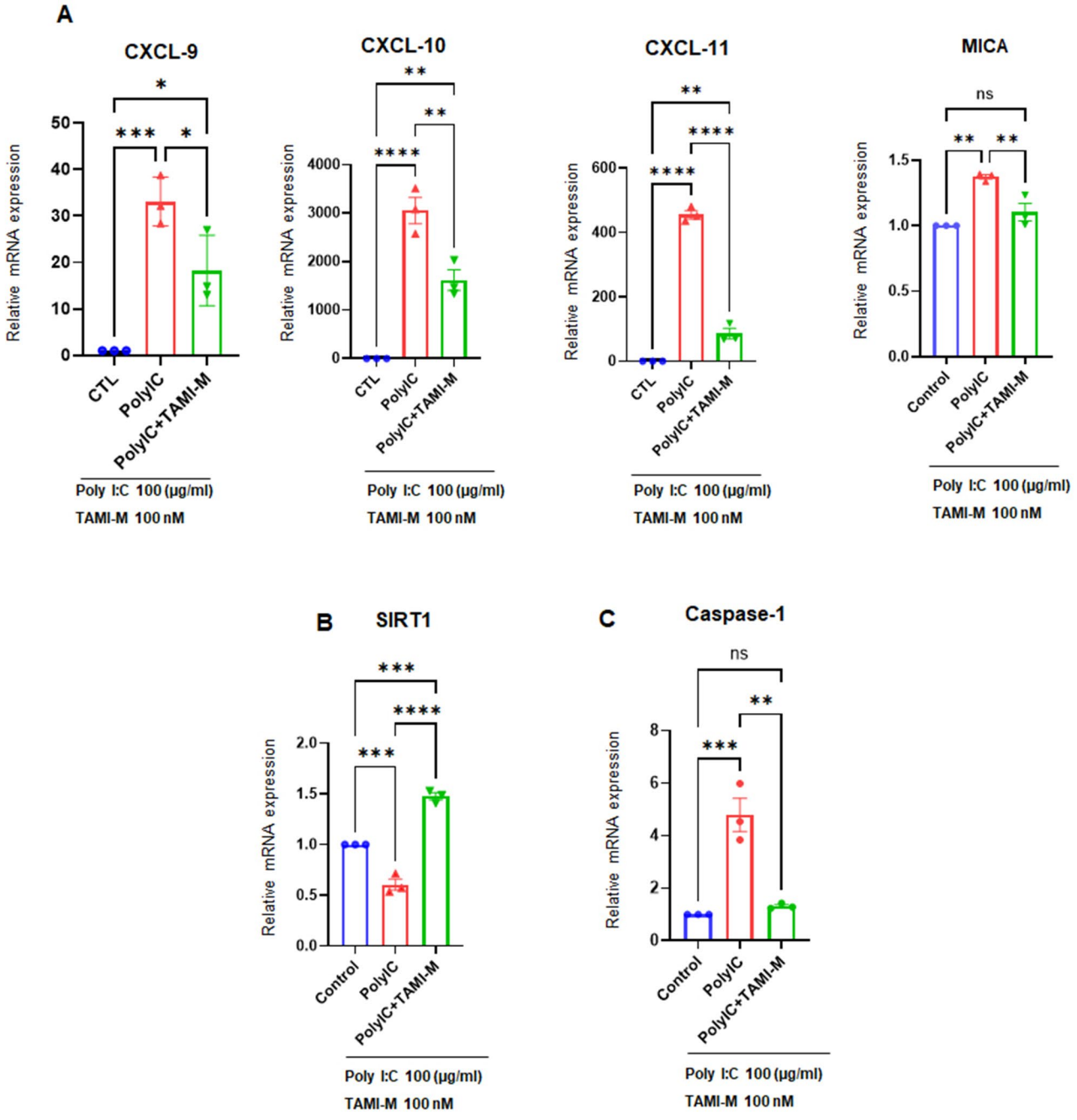

Fig. 3 Effect of TAMI-M on Poly I: C-induced production of proinflammatory chemokines, SIRT1, and Caspase-1 in HORS: **A** HORS cells were treated with Poly I: C for 3 h to simulate an inflammatory environment resembling alopecia areata pathology. TAMI-M was applied to evaluate its potential effects. Relative mRNA expression levels of proinflammatory chemokines were quantified using qPCR. Treatment with TAMI-M significantly reduced chemokine production compared to Poly I: C-induced controls. **B** TAMI-M treatment restored SIRT1 expression levels, which were downregulated following Poly I: C induction. **C** Caspase-1 activity, a marker of inflammasome activation, was measured by qPCR. TAMI-M treatment significantly reduced Caspase-1 activity in HORS cells compared to Poly I: C-treated controls. Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). Significant differences are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between groups

cytokine levels. These cytokines contribute to the inflammation observed in AGA, particularly through AR mediated pathways.

Furthermore, western blot analyses revealed that DHT treatment significantly increased inflammatory infiltrates and the expression of pro-inflammatory cytokines in the skin. However, TAMI-M treatment resulted in a significant reduction in both the inflammatory infiltrates and cytokine expression, illustrating its potent anti-inflammatory properties (Fig. 5B, C). These results suggest that

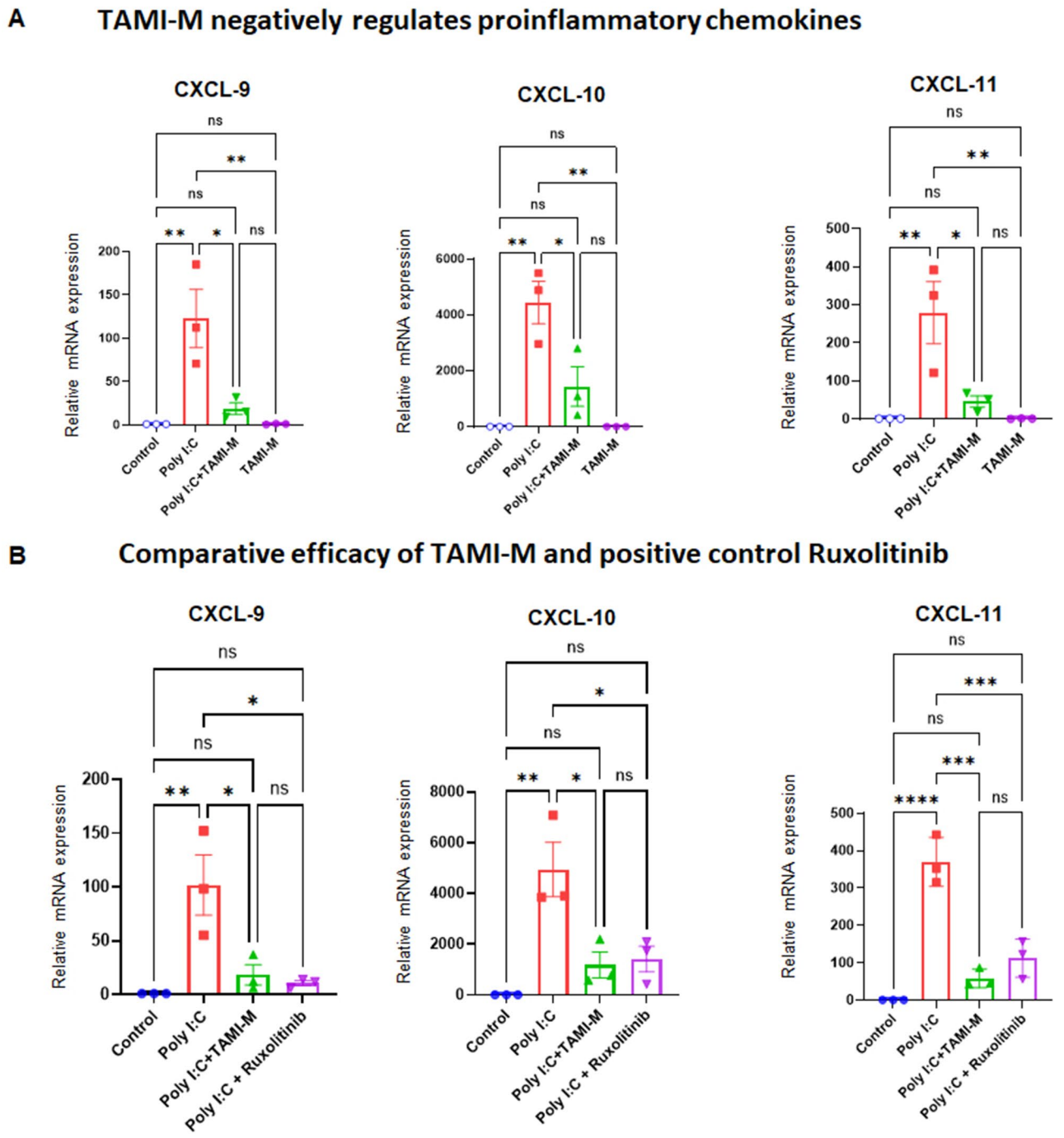

Fig. 4 Effects of TAMI-M on Polyinosinic: polycytidylic acid-induced production of proinflammatory chemokines in HORS Cells. **A** mRNA expression levels of CXCL9, CXCL10, and CXCL11 in human ORS cells treated with Poly I: C (10 µg/mL) and TAMI-M (100 nM) for 24 h. **B** mRNA expression levels of CXCL9, CXCL10, and CXCL11 in human ORS cells treated with Poly I: C, TAMI-M, and the positive control Ruxolitinib (100 nM) for 24 h. Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). Significant differences are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between groups. This figure illustrates the differential impact of TAMI-M and Ruxolitinib on the expression of chemokines associated with AA pathogenesis

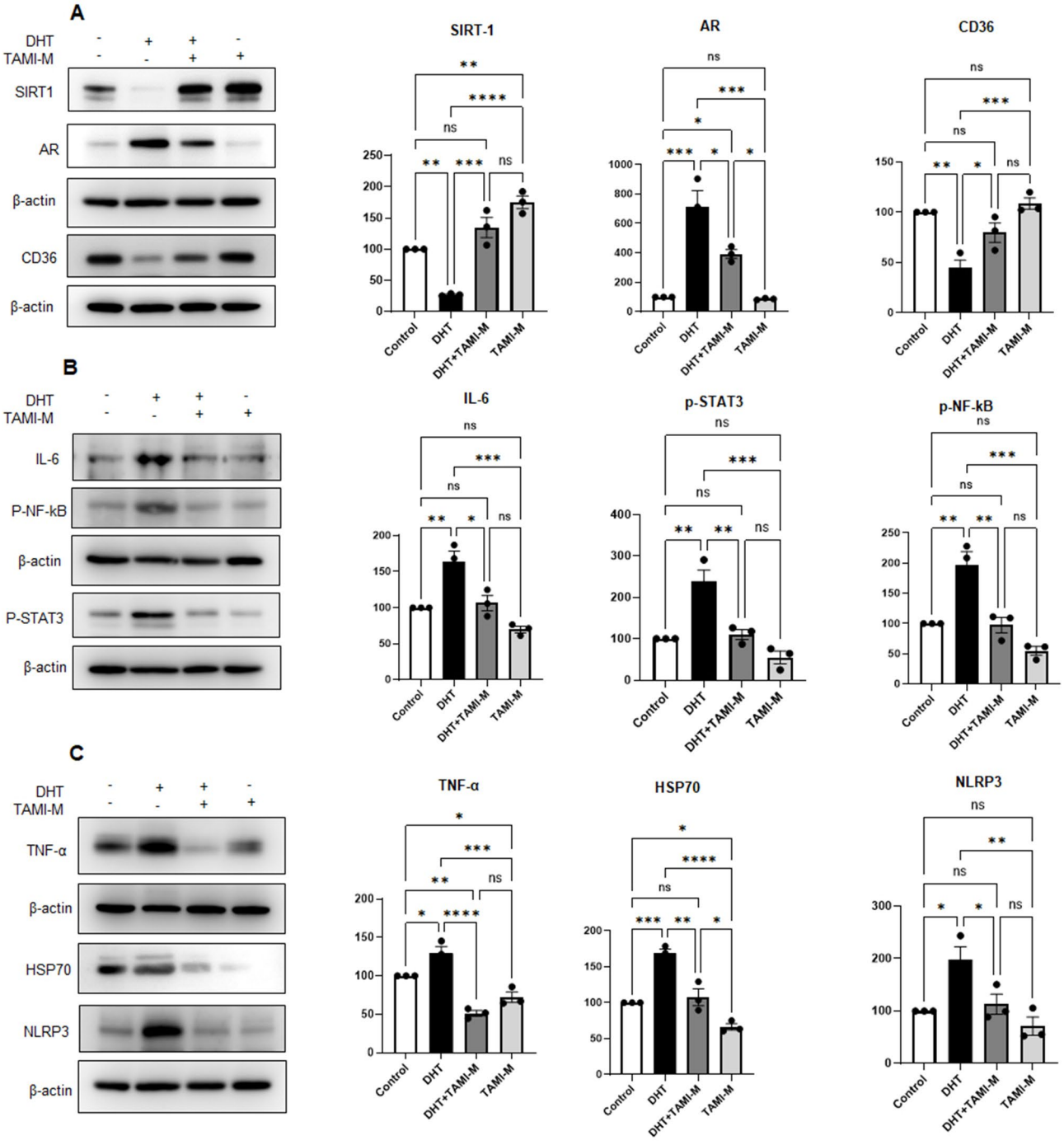

Fig. 5 The counteractive effect of SIRT1 on NF-κB signaling by TAMI-M in vivo. **A** Immunoblotting analysis of SIRT1, AR, and CD36 expression in control, DHT-induced AGA mice, and TAMI-M treated mice (2 × 0.6 mg/kg, subcutaneously). **B** Expression of inflammatory markers: IL-6, P-STAT3, and P-NF-κB in the same groups. **C** Expression of TNF-α, HSP70, and NLRP3 in the control, DHT-induced AGA, and TAMI-M treated groups. Relative protein expression levels were quantified and normalized to β-actin. Individual data points are shown to illustrate variation within groups. Statistical significance was determined using one-way ANOVA followed by post hoc comparisons (Tukey's test). Significant differences are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

TAMI-M can effectively modulate the inflammatory environment associated with AGA, offering a promising therapeutic approach.

We investigated the association between HSP70 expression and AGA, recognizing its role in stress responses, including those induced by androgen signaling. Our results demonstrated that DHT treatment significantly upregulated the expression of HSP70, TNF-α, and NLRP3 (Fig. 5C). Conversely, treatment with DHT + TAMI-M markedly suppressed the expression of these

markers compared to the control group (Fig. 5C), highlighting the therapeutic potential of miR-485-3p ASO in mitigating DHT-induced inflammatory responses.

Discussion

In this study, we established an AA model using C3H/HeJ mice via Poly(I: C) and IFN-γ injections, mimicking the clinical manifestations of human AA. TAMI-M treatment significantly reduced lesion areas, with weekly administration showing a more pronounced effect, suggesting its potential as a therapeutic agent. AA pathogenesis is characterized by immune dysregulation and inflammation, with upregulated CXCL9, CXCL10, and CXCL11 as key markers [3, 16, 20, 21]. our study demonstrated that the experimental AA model induced by Poly (I: C) and IFN-γ resulted in elevated levels of chemokines and cytokines, including CXCL-9, CXCL-10, CXCL-11, and IFN-γR, in affected lesion area. These findings align with previous studies highlighting the critical role of inflammation in AA pathogenesis [16, 21].

Furthermore, our study revealed that TAMI-M treatment significantly attenuated the expression of these proinflammatory mediators, suggesting its potent role of anti-inflammatory properties. Importantly, the observed downregulation of proinflammatory cytokines and chemokines by TAMI-M suggests a potential mechanism of action involving the modulation of immune responses within the hair follicle microenvironment. These findings are consistent with previous studies demonstrating the role of inflammation in hair loss disorders and also shown promising results in other autoimmune related diseases as well [24–26]. The anti-inflammatory effects of TAMI-M observed in this study contribute to its therapeutic efficacy in promoting hair regrowth and reducing AA lesion size. These findings support the potential of TAMI-M as a promising therapeutic candidate for the treatment of AA.

To further investigate the effects of TAMI-M on proinflammatory responses in human hair follicles, we utilized human ORS cells. Stimulation of ORS cells with poly(I: C) induced a significant increase in the expression of proinflammatory chemokines (CXCL9, CXCL10, CXCL11, and MICA). CXCL9, CXCL10, and CXCL11 stand out as potential biomarkers for autoimmune diseases, given their involvement in orchestrating inflammatory responses. Our study delved into the role of TAMI-M and it's impact on these proinflammatory chemokines in human ORS cells. Impressively, the TAMI-M showcased a robust downregulation of CXCL9, CXCL10, and CXCL11, hinting at their therapeutic potential in addressing autoimmune conditions. Importantly, the anti-inflammatory effects of TAMI-M were comparable to those of the established drug, Ruxolitinib, suggesting its potential as a promising therapeutic candidate for AA. Since Ruxolitinib is known to be a JAK1/2 inhibitor, it completely blocked phosphorylation of JAK1 and 2 [3, 27–29]. While our study primarily focused on the role of miR-485-3p in the inflammatory mechanisms of AA, as it is crucial to compare with established therapies like Ruxolitinib. Our future investigations will include these comparisons to evaluate the relative effectiveness, safety, and convenience of miR-485-3p modulation. Additionally, incorporating proteomic profiling will provide deeper insights into the mechanisms of both established and new therapies, enhancing our understanding of their impact on hair follicle recovery and inflammation. Thus, the observed downregulation of proinflammatory chemokines by TAMI-M aligns with its therapeutic efficacy in the mouse model of AA, further supporting its role in mitigating hair loss.

Earlier study utilizing an AGA mouse model given the established role of inflammation in AGA pathogenesis (unpublished data), further we investigated the potential involvement of the NLRP3 inflammasome in this process. Our findings revealed that in the AGA mouse model, NLRP3, IL6, IL-1β, and Caspase-1 expression were significantly upregulated, suggesting the inflammasome activation. These results are consistent with previous studies implicating NLRP3 inflammasome activation in the pathogenesis of AA diseases, autoinflammatory and autoimmune diseases [19, 21, 30, 31]. Moreover, in our current study, we observed HSP70 expression by TAMI-M, support its broader therapeutic potential in inflammatory and various autoimmune diseases conditions like AA [32–36].

The observed downregulation of immune-inflammatory components by TAMI-M further supports its anti-inflammatory properties and its potential as a therapeutic agent for AGA as well as AA. By targeting key inflammatory pathways, TAMI-M may offer a novel therapeutic strategy for managing AGA and immune modulatory diseases like AA.

To the best of our knowledge, there are currently no clinically approved therapies that specifically target SIRT1 for the treatment of AA. However, preclinical data on SRT1720 [16, 37], a selective SIRT1 activator, suggests its potential to modulate inflammatory stress. Although SRT1720 has not been directly evaluated for AA, its ability to influence inflammatory pathways underscores the therapeutic relevance of targeting SIRT1 in inflammatory conditions. Our study highlights the pivotal role of SIRT1 activation in AA and addresses a critical gap in the development of targeted therapies for this condition.

While pro-inflammatory cytokines such as IL-1β and TNF-α are also important mediators of inflammation, we recognize that their inclusion in our analysis would have provided a broader view of the inflammatory landscape. However, given the specific focus of our study, we decided

to prioritize the chemokines most directly related to the IFNγ pathway. IL-1β and TNF-α are acknowledged as central to the inflammatory cascade in various diseases, including AA and Alzheimer's Disease (AD), as demonstrated by previous studies [16, 17, 19]. Koh et al. (2021) demonstrated that miR-485-3p ASO treatment reduced the secretion of pro-inflammatory cytokines such as IL-1β and TNF-α, highlighting the potential therapeutic effects of miR-485-3p modulation in inflammatory diseases like AD. Similarly, studies have showed that SIRT1 downregulation, which provokes immune-inflammatory responses, leads to elevated expression of IL-1 and TNF-α, contributing to an unfavorable microenvironment in diseases such as AA [16].

Translating miRNA therapeutics like miR-485-3p ASO into clinical practice presents several challenges, including efficient in vivo delivery, minimizing off-target effects, and ensuring sustained therapeutic efficacy. Our study addressed these through a lipid-based delivery system optimized for subcutaneous administration, achieving effective tissue penetration and target modulation. However, further research is needed to evaluate long-term safety, efficacy, and pharmacokinetics in preclinical models.

Preclinical models, while valuable, often fail to fully replicate human immune responses and disease heterogeneity, complicating predictions of therapeutic performance in humans. Safety concerns, including off-target effects and immunogenicity, remain critical hurdles for nucleic acid-based therapies. Additionally, scalable manufacturing and regulatory compliance are essential for clinical translation. Our findings suggest promising anti-inflammatory and hair regrowth effects, highlighting its potential as a novel therapy for AA and AGA. Addressing these translational challenges systematically will facilitate progress toward clinical application.

Conclusion

Mechanistically, our findings indicate that TAMI-M exerts its beneficial effects through multiple pathways, including the modulation of immune responses, reduction of inflammation, and potentially the regulation of key signaling pathways such as NF-κB and STAT3. Further investigation incorporating larger cohorts into the precise molecular mechanisms underlying the therapeutic effects of TAMI-M, particularly its interaction with immune checkpoint inhibitors or JAK inhibitors. These interactions will highlight its role in mitigating inflammation and autoimmune-driven hair loss.

Abbreviations

AA	Alopecia areata
IFN-γ	Interferon-gamma
Poly(I:C)	Polyinosinic-polycytidylic acid
SIRT1	Sirtuin 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
CXCL	C-X-C motif chemokine ligand 1
STAT3	Signal transduction and activator of transcription 3
ASO	Antisense oligonucleotides
AGA	Androgenic alopecia
ORS	Human outer root sheath cell
EDTA	Ethylenedia-minetetraacetic acid
IL-6	Interleukin-6
TNF-α	Tumor Necrosis Factor Alpha
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
AR	Androgen receptor

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

Jin-Hyeob Ryu: Conceptualization, project administration and coordination, study design contributions, and overall supervision. Hyun-Jeong Cho: Project administration and coordination, study design contributions, and personnel supervision. Insun Kim: Design and synthesis of ASO (TAMI-M), Hyun Su Min: Methodology support. Yu Na Lim: Methodology support. Hossain Md Jamil: execution of the in vivo studies (experiments and data analyses), support in manuscript writing, and review. Begum Shahnaz: Design and execution (experiments and data analyses) of studies; research and management team reviews; manuscript writing and review. The author(s) read and approved the finalmanuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Some data may not be available owing to privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate

The animal experimentation conducted in this study adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of Biorchestra (permit numbers: BOIACUC-20230531-0002, BOIACUC-20220908-0001).

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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