Ultraviolet Light-Emitting-Diode Irradiation Inhibits TNF- α and IFN- γ -Induced Expression of ICAM-1 and **STAT1** Phosphorylation in Human Keratinocytes

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Background and Objectives: Ultraviolet light-emitting diodes (UV-LEDs) are a novel light source for phototherapy. This research investigated the in vitro safety and efficacy of UV-LEDs as a phototherapeutic device for atopic dermatitis (AD).

Study Design/Materials and Methods: Human keratinocytes and fibroblasts were irradiated by UV-LEDs with a center wavelength of 310 and 340 nm. We examined the effects of UV-LED irradiation on the suppression of TNF- α / IFN-y-induced activation of STAT1 and ICAM-1 and on NF-KB expression; we used the following methods: cell viability assay, reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay, Western blotting, and immunocytochemistry.

Results: We observed anti-inflammatory responses through the suppression of TNF- α /IFN- γ -induced expression of TARC and MCP-1/CCL2, IL-1beta, IL-6, and sICAM-1 via blockage of ICAM-1 activation and subsequent activation of STAT1 and NF-KB. The results suggested that UV-LED irradiation inhibited ICAM expression by suppressing TNF-α/IFN-γ-induced NF-κB activation in vitro.

Conclusion: We concluded that novel UV-LED (310 and 340 nm) modalities were effective for the treatment of AD and may be promising for the treatment of inflammatory skin diseases. Lasers Surg. Med.

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Key words: UV-LEDs; atopic dermatitis; HaCaT

INTRODUCTION

inflammatory reaction. Keratinocytes produce cytokines and chemokines, are actively involved in the skin immune system, and are crucial for AD pathogenesis [3]. TNF- α and IL-1 are produced by keratinocytes, mast cells, and dendritic cells upon nonspecific stimulation such as scratching [4]. These cytokines subsequently bind to receptors on the endothelium to induce the expression of adhesion molecules, which facilitates the migration of inflammatory cells from the peripheral blood into the tissue [5]. Thymus and activation-regulated chemokine/ CCL17 (TARC) is increased in human atopic lesional skin and may attract Th2 cells expressing the TARC-sensitive chemokine receptor CCR4 [6]. The TARC chemokine is involved in lymphocyte migration. Migration of inflammatory cells into the skin is facilitated by chemokines produced in the skin [7]. In addition, IL-31 is implicated in inflammatory and lymphoma-associated itching [8], and IL-31-induced keratinocytes produce IL-20 and IL-24, which regulate proliferation and differentiation during inflammation and seem to disrupt the skin barrier [9]. Therefore, reduction of itch-related endogenous mediators is the most useful therapeutic strategy for mitigating AD skin lesions.

The most common phototherapy for skin disease uses narrowband ultraviolet B (UVB) light [10]. Ultraviolet light sources can successfully treat psoriasis and are promising for treating other skin diseases including acne vulgaris, AD, and vitiligo [11]. The effectiveness of narrowband UVB (NBUVB: 311-313 nm) and high-dose

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Atopic dermatitis (AD) is a frequently occurring inflammatory skin disease associated with severe itching and allergies to environmental factors [1]. AD is characterized by the predominant infiltration of Th2-type cells and increased secretion of Th2-related cytokines [2]. In the chronic phase, Th1 cells also infiltrate lesional skin and are involved in AD pathogenesis. Many mediators affect the AD

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UVA1 (340–400 nm) over previously used approaches (broadband UVB and PUVA) for treating AD is clear. Topical steroids are the most effective drugs for achieving quick remission of inflammation and have been used for decades [12]. Topical treatments are used first as they may have substantial adverse effects such as skin atrophy, telangiectasia, and hypertrichosis. Patients with AD have limited therapeutic options such as oral steroids, cyclosporine A, or phototherapy. Therefore, new treatment modalities are required.

UV-LEDs emit radiation in the UVA and UVB ranges (approximately 300–350 nm) [13]. An advantage of UV-LEDs is that they can be fabricated so the emission wavelength covers the required range of treatment while avoiding the erythema threshold. In the near future, UV lamps will be changed to UV-LEDs because of their small size, low power consumption, high reliability, and health and safety benefits. These devices have the potential to be used in a variety of applications including UV curing [14], biomedicine [15], and a range of phototherapy applications [16,17] such as the treatment of skin conditions. However, the underlying mechanisms of UV-LEDs-induced biological actions are not clear.

Exposure of keratinocytes to interferon (IFN- γ) and tumor necrosis factor (TNF- α) leads to abnormal secretion of cytokines and chemokines [18]. NF- κ B is activated when cells are stimulated with TNF- α and IFN- γ , which are involved in the activation of responsive genes [19]. Signal transducers and activators of transcription 1 (STAT1) are pivotal regulators of the IFN- γ -induced immune response in keratinocytes [20]. Cytokine-induced ICAM-1 expression may be efficiently inhibited by irradiation of cultured keratinocytes with sublethal doses of UVB or UVA [21]. NBUVB phototherapy has been used in both animal [22] and human studies [23]. However the UV-LED treatment mechanism is still unknown.

We evaluated a new phototherapy device that uses UV-LEDs to treat inflammatory skin disease. UV-LEDs attenuated inflammatory cytokine release and suppressed phosphorylated STAT1 by inhibiting IFN- γ -signaling in keratinocytes. From our findings, we concluded that phototherapy devices are possible candidates for AD treatment.

MATERIALS AND METHODS

Cell Culture and LED Treatment

The human keratinocyte cell line HaCaT and the human dermal fibroblast cell line CCD-25SK were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator under a 5% CO₂ atmosphere. Cells were pretreated with 100 ng/ml TNF- α and 100 ng/ml IFN- γ for 1 hour and radiated with UV-LEDs at the indicated energy level. Cells were used after 24 hours. LED devices were provided by the Seoul Viosys Institute (Ansan, Korea). HaCaT cells were irradiated using a novel UV-LED device for *in vitro* testing (130 × 90 mm). Power fluences of 310 nm (171 µW/cm²) or

 $340 \text{ nm} (7.44 \text{ mW/cm}^2)$ were delivered to a platform 50 mm under the LED arrays. Before UV-LED (310 and 340 nm) irradiation, the cultures were rinsed in PBS and irradiated with the indicated fluencies in PBS to avoid absorption by the phenol-red in the culture medium. All groups were kept in PBS at room temperature during the experimental procedure to ensure equal treatment conditions.

Isolation of Nuclear and Cytoplasmic Proteins

Nuclear and cytoplasmic proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit following provided instructions (Pierce Biotechnology, Rockford, IL). Protease inhibitor tablets (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors 10 mM sodium pyrophosphate and 100 μ M sodium orthovanadate were added to extraction reagents before use.

Immunocytochemistry

HaCaT cells at 2.0×10^4 cells/500 µl chamber were seeded onto chamber slides and irradiated with UV-LEDs. After 24 hours, cells were treated with 4% paraformaldehyde for 10 minutes and 0.1% Triton X-100 for 5 minutes. Cultured HaCaT cells were incubated with anti-NF- κ B/p65 (1:500, ab7970, Abcam, Cambridge, UK) at 4 °C overnight and with FITC-labeled goat anti-rabbit IgG (1:1,000, NB730-F, Novus Biologicals, CO). DAPI mounting media kits were used to counterstain nuclei. Immunostained cells were mounted with medium containing DAPI and visualized using an Olympus FLUOVIEW FV10i confocal microscope.

Western Blotting

Protein extracts were prepared using RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% DOC, 1mM PMSF, 25mM MgCl₂, and phosphatase inhibitor cocktail). Amounts of protein in extracts were quantitated using a Bio-Rad DC Protein Assay Kit II (Bio-Rad, Hercules, CA). After quantitation, equal amounts of protein were resolved on 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes. After blocking with 5% nonfat milk, membranes were probed with specific antibodies against tyrosine 701 (pY-STAT1) (1:1,000, 9167, Cell Signaling Technology, Danvers, MA), serine 727 (pS-STAT1) (1:1,000, 9177, Cell Signaling Technology), total STAT1 (1:1,000, 9172, Cell Signaling Technology), ICAM-1 (1:1,000, ab2213, Abcam, Cambridge, MA), or β -actin (1:1,000, 4967, Cell Signaling Technology). After washing, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies. Immunoreactive signals were detected using enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ). Protein levels were compared to loading controls.

Reverse Transcription-PCR Analysis

Total RNA was purified using the RNeasy Plus mini kit (Qiagen, Valencia, CA) following the protocol of the manufacturer. One microgram of DNase-treated total RNA was used for first-strand cDNA. This reaction was performed using a random primer and M-MLV reverse transcriptase (Invitrogen Corp., Carlsbad, CA). The primers for reverse transcription-PCR (RT-PCR) are as follows. *ICAM-1*: sense CAC CCT AGA GCC AAG GTG AC – 3' and antisense 5' – CAT TGG AGT CTG CTG GGA AT – 3'. The general PCR conditions were 30–35 cycles at 94 °C for 2–10 minutes, 94 °C for 30 seconds–3 minutes, 50–58 °C for 30 seconds–1 minutes, 72 °C for 30 seconds–1 minutes, and 72 °C for 4–7 minutes. The PCR products were run on 0.75–1.5% agarose gels. Images of the gels were analyzed by Quantity One software (Bio-Rad).

Measurement of Inflammatory Cytokines

IL-1b, IL-6, MCP-1/CCL2, TARC, and sICAM levels were measured in cell supernatants using human enzymelinked immunosorbent assay (ELISA) kits (eBioscience San Diego, CA). All analyses were according to the manufacturer's protocol.

Statistical Analyses

All experiments were repeated at least three times. Results are reported with standard deviation (SD). Statistical significance was determined by one-way AN-OVA followed by Duncan's multiple range tests and results reported as means \pm standard error of the mean (SEM). *P*-values of **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 were considered statistically significant.

RESULTS

Effect of UV-LED Radiation on Viability of Human Keratinocytes

To determine an irradiation range for investigating the effects of UV-LEDs on HaCaT cell viability, we used 310nm UV-LED irradiation at $171 \,\mu\text{W/cm}^2$ and radiant exposures of 5, 10, 20, and 50 mJ/cm² and 340-nm UV-LED irradiation at 7.44 mW/cm² and radiant exposures of 1, 2, 5, and 7 J/cm². Cell viability determined by CCK-8 assays are in Figure 1. Proinflammatory cytokines, such as IFN- γ and TNF- α , mimic inflammatory conditions such as AD. As shown in Figure 1, 310-nm irradiation from 5 to 50 mJ/cm² had no significant cytotoxic effects after 24 hours on HaCaT keratinocytes; however, costimulating cells with TNF- α (100 ng/ml) and IFN- γ (100 ng/ml) for <1 hours after 310-nm irradiation had significant cytotoxic effects at 50 mJ/cm² on HaCaT keratinocytes. However, 340-nm irradiation from 1 to 5 J/cm² had no significant cytotoxic effects after 24 hours on HaCaT keratinocytes but 7 J/cm² of 340-nm irradiation had significant cytotoxic effects. We found that 340-nm UV-LED more strongly inhibited the growth of inflammatory-conditioned cells. Early apoptotic changes in cell shape and morphology were monitored and 340 nm UV-LED-irradiated CCD25SK fibroblasts showed biophysical and apoptotic morphological changes (Data not shown). Thus, 310-nm UV-LED radiant exposures of

 $5-50 \text{ mJ/cm}^2$ and 340-nm UV-LED $1-5 \text{ J/cm}^2$ exposures were used in subsequent experiments.

UV-LED Suppresses TNF-α/IFN-γ-Induced ICAM-1 Expression and STAT1 phosphorylation in Human Keratinocytes

Next, we investigated the effects of UV-LEDs irradiation on signaling cascades downstream of STAT1, which is a molecular link between inflammation and TLR signal transduction [24]. We tested whether TNF- α and IFN- γ induced dose-dependent and time-dependent STAT1 phosphorylation in HaCaT keratinocytes (Fig. 2a and b). Inflammatory mediators increased STAT1 phosphorylation at Tyr 701 and Serine 727 at 5 minutes after stimulation with 100 ng/ml each TNF- α and IFN- γ , and p-STAT1 remained at maximum levels at 180 minutes. Treatment with TNF- α and IFN- γ increased total phosphorylated STAT1 dose-dependently for 24 hours. Cells were pretreated with TNF- α and IFN- γ for 1 hours and irradiated at 310 nm (5, 10, 20, and 50 mJ/cm²) or 340 nm (1, 2, 5 J/cm²) for 24 hours.

Intercellular adhesion molecule-1 [ICAM-1] depends on an IFN- γ signal transduction pathway with STAT1 transcription factor as an intermediate. We analyzed for expression of p-STAT1 and ICAM-1 by Western blot. Tyrosine 701-phosphorylation of STAT1 decreased with 340-nm, 2J/cm² irradiation, whereas beta-actin protein did not decrease (Fig. 2c and d).

Next, we investigated the changes in the expression of *ICAM-1* genes by performing reverse transcription polymerase chain reaction (RT-PCR) in HaCaT cells. Interestingly, *ICAM-1* expression levels were decreased in a dose-dependent manner when cells were irradiated with 310 nm (10, 20 mJ/cm²) or 340 nm (1, 2 J/cm²) for 24 hours (Fig. 2e). These results suggested that 310 nm and 340 nm UV-LED suppressed STAT1 signaling and inhibited inflammation by downregulation of ICAM-1.

Effects of UV-LED on TNF- α /IFN- γ -Induced Expression of Chemokines/Cytokines in HaCaT Cells

To determine whether the TNF- α and IFN- γ induction of inflammation correlated with cytokine production, we exposed HaCaT keratinocytes to TNF- α and IFN- γ and did or did not irradiate with UV-LED before assessing IL-1b, IL-6, MCP-1/CCL2, TARC, and sICAM-1 production by ELISA. Cells exposed to TNF- α and IFN- γ had elevated levels of IL-1b, IL-6, MCP-1/CCL2, TARC, and sICAM-1 in the medium after 24 hours. We found significant decreases in IL-1b (by 46%), MCP-1/CCL2 (by 18%), TARC (by 27%), and sICAM-1 (by 40%) in cells irradiated with 2 J/cm² and 340 nm. However, no significant difference was seen in IL-6 levels (Fig. 3). ICAM-1 exists in a soluble form (sICAM-1) that proteolytically cleaves full-length ICAM-1 near its transmembrane region; its level is elevated in AD [25]. Co-irradiation with 20 mJ/cm², 310 nm and 2 J/cm², 340 nm UV-LED did not have synergistic effects. However, additional affects



Fig. 1. Effects of 310-nm and 340-nm UV-LEDs on HaCaT human keratinocyte viability. Cytotoxicity of UV-LEDs to HaCaT cells was measured using CCK-8 assays. Cells in 96-well microplates (2 \times 10⁴/well) were irradiated with 310 nm (5, 10, 20, 50 mJ/cm²), 340 nm (1, 2, 5, 7 J/cm²), or 310 nm and 340 nm from UV-LEDs for 24 hours (black bars). Cells (2 \times 10⁴/wells) were also pretreated with TNF- α (100 ng/ml) and IFN- γ (100 ng/ml) for 1 hour before radiation (gray bars). Unirradiated cells were the control group. The 310-nm irradiated groups were stimulated with 171 μ W/cm². The 340-nm irradiated groups were stimulated with 7.44 mW/cm². Values are mean \pm SD of three independent experiments.

of UV-LED wavelength might contribute to additive effects on downregulation of sICAM-1 release. Therefore, co-irradiation might decrease the release of sICAM-1 and attenuate inflammatory reactions. These results suggested that 310 and 340 nm UV-LED exerted immunosuppressive and anti-inflammatory effects.

Effects of UV-LED on TNF- α /IFN- γ -Induced Activation of NF- κ B and STAT1 in HaCaT Cells

We studied the effects of UV-LED irradiation that led to the expression of NF- κ B in TNF- α and IFN- γ -stimulated HaCaT cells. NF- κ B and STAT1 are involved in the initiation of the inflammatory cascade and their activation induces production of proinflammatory cytokines and chemokines. Therefore, we examined the inhibitory effects of UV-LED irradiation on the NF- κ B signaling pathway in TNF- α and IFN- γ -stimulated HaCaT cells. Nuclear and cytosolic extracts were evaluated by immunoblotting for NF-κB activation. Treatment with 340-nm (2 J/cm²) UV-LED inhibited TNF- α and IFN- γ -induced nuclear translocation of NF-KB/p65 (Fig. 4a). A combination of 310-nm and 340-nm irradiation had weak synergistic effects on the decrease of phosphorylated STAT1 and ICAM-1 on HaCaT cells treated with TNF- α and IFN- γ (Fig. 4c). We also confirmed the effect of UV-LED irradiation on nuclear translocation of NF-KB/p65 by immunocytochemistry. NFκB/p65 in the cytosol was translocated to the nucleus after TNF- α and IFN- γ stimulation and this was inhibited by $340 \,\mathrm{nm} \, (2 \,\mathrm{J/cm}^2)$ (Fig. 4e). These observations suggested that inhibition of TNF-a/IFN-y-induced production of chemokines (TARC, MDC-1/CCL2) and inflammatory cytokines (IL-1b, sICAM-1) by UV-LED irradiation was mediated by suppression of NF-KB and STAT1 expression.



Fig. 2. Effects of 310-nm and 340-nm UV-LEDs on TNF-α/IFN-γ-induced NF-κB and STAT1 activation in HaCaT cells. (a) Cell lysates were prepared and phosphorylation of tyrosine 701 (pY-STAT1) or serine 727 (pS-STAT1) was determined in cells stimulated by TNF-a (100 ng/ml) and IFN-γ (100 ng/ml) for 0, 5, 10, 30, 60, 120, 180 minutes. (b) Dose dependence. STAT1 phosphorylation and STAT1 and β -actin levels were determined by immunoblotting of whole-cell lysates. (c) Cells were pretreated with TNF- α (100 ng/ml) and IFN- γ (100 ng/ml) for 1 hour and irradiated with UV-LEDs at indicated energy levels for 24 hours. Cell lysates were prepared and subjected to Western blotting for pY-STAT1, pS-STAT1, ICAM-1, β -actin. (d) Relative ratios of proteins were determined as band intensities with ratios of pY-STAT1, pS-STAT1, ICAM-1 in each irradiation group relative to the control. (e) RT-PCR analysis of ICAM-1 and GAPDH expression using total RNA prepared from UV-LEDs irradiated HaCaT cells. (f) ICAM-1 Expression was normalized for the amount of RNA loaded per lane by dividing by the optical density of GAPDH. Data represent three independent experiments. Significant differences from the control group are *P < 0.05; **P < 0.01; ***P < 0.001 versus TNF- α /IFN- γ treatment plus irradiation.

(a) 30.00

0.00 IT-1peta (bg/ml) 15.00 5.00 0.00

NS



340-1 J

310-20 mJ 310-20 mJ

340-2 J



Fig. 3. Effects of UV-LED (310, 340 nm) on TNF- α /IFN- γ -induced IL-1b, IL-6, MCP-1/CCL2, TARC, and sICAM production in HaCaT cells. Cells were pretreated with TNF- α (100 ng/ml) and IFN- γ (100 ng/ml) for 1 hour and irradiated with UV-LEDs. Production of (a) IL-1b, (b) IL-6, (c) MCP-1/CCL2, (d) TARC, and (e) sICAM were measured by enzyme-linked immunosorbent assays of cell supernatants. Values are mean \pm SD of three independent experiments. Significant differences from the control group are *P < 0.05; **P < 0.01; ***P < 0.001 versus TNF- α /IFN- γ treatment alone.

MODULATION OF ICAM-1 IN HACAT BY UV-LEDS





DISCUSSION

AD is a common inflammatory skin disorder characterized by abnormalities in both skin barrier structures and alterations in the immune response [26]. Currently, AD treatment is based on an individualized approach using topical corticosteroids and immunomodulators, emollients, phototherapy, and antibiotics. immunosuppressive agents [27]. Phototherapy is the controlled use of UV light to treat skin conditions, including AD. Typically, UVB, UVA, or a combination of UVB and UVA are used [28]. These regimens are considered a second-line treatment for AD, especially for adults with AD that is not adequately managed with topical corticosteroids, topical calcineurin inhibitors, or oral corticosteroids. The long-term effects of phototherapy include increased risk of skin cancer; shortterm adverse effects include itching and acute burns [29]. Nevertheless, many studies have shown that phototherapy targets inflammatory cells and changes inflammatory cytokine production in vitro. Therefore, we evaluated novel 310-nm and 340-nm UV-LED phototherapy inhibition of AD-like symptoms and pathogenesis using in vitro models.

In AD, keratinocytes produce chemokines and cytokines following mechanical stimulation. TNF- α and IFN- γ synergistically induce crucial cytokines that cause AD symptoms in keratinocytes. This experimental model has been widely used to mimic AD symptoms *in vitro*. The UV-LED irradiation doses (5–20 mJ/cm2 of 310-nm UV-LED and 1–2 J/cm2 of 340-nm UV-LED) we used did not affect cell viability.

Our in vitro data showed the effects of novel UV-LEDs (310 and 340 nm) on TNF- α /IFN- γ -stimulated HaCaT cells. Aragane et al. [21] demonstrated that UVR inhibits IFN-signaling by blocking phosphorylation of STAT1, suggesting that activation of a phosphatase might be involved. Direct induction of SOCS molecules by UV light probably results in the immunosuppressive effects of UV phototherapy. STAT1 has a positive role in early-stage inflammation-associated diseases by accelerating inflammation. Thus, STAT1 promotes inflammation-mediated disease progression. If the immunosuppressive effects of UV phototherapy are through regulation of SOCS expression via IFN-induced Jak/STAT signaling, these molecules may be a new drug targets [30]. Our results suggested that 340-nm UV-LED effectively suppressed STAT1 signaling and inhibited inflammation through downregulation of ICAM-1.

We found reduced IL-1b, MCP-1/CCL2, TARC, sICAM-1 levels in HaCaT cells irradiated at 310 and 340 nm. IL-1 promotes expression of adhesion molecules on keratinocytes and endothelial cells, allowing the infiltration of inflammatory. In addition, release of IL-1 from the epidermis after activation is a primary event in promotion of inflammatory skin conditions through induction of proinflammatory cytokines and adhesion molecules such as ICAM-1 [31]. Adhesion molecules are enhanced in acute and chronic AD lesions [32,33]. Thus, ICAM-1 is a potential therapeutic target for modulating AD. NF-κB is critical for the regulation of cell survival genes and coordinates the expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [34]. Since the expression of these proinflammatory mediators is modulated by NF-κB, our findings suggested that inhibition of TNF-α and IFN-γ-induced nuclear translocation of NF-κB/ p65 and inhibition of the transcription of proinflammatory mediators IL-1beta, IL-6, sICAM-1, and TARC by UV-LED irradiation occurred by blocking the STAT1/ICAM-1/NFκB signaling pathway. In summary, our *in vitro* data showed that UV-LEDs significantly inhibited TNF-α/IFNγ-induced NF-κB activation and STAT1 activation.

We hypothesized that AD treatment could be directed to control scratching behavior, suppress inflammation, and improve skin barrier function. Although further study is needed on the action mechanism of UV-LED irradiation *in vivo*, the therapeutic effect of UV-LEDs may be through effects on itching, inflammation suppression, and restoring the skin barrier.

In conclusion, the results in this report demonstrated that 310-nm and 340-nm UV-LED irradiation inhibited ICAM expression by suppressing TNF- α /IFN- γ -induced NF- κ B activation in HaCaT cells. From these results, we suggest that 310-nm and 340-nm UV-LED phototherapy might be a therapeutic strategy for AD treatment.

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