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ORIGINAL ARTICLE

Effects of Dexamethasone and Tacrolimus on Mesenchymal Stem Cell Characteristics and Gene Expression

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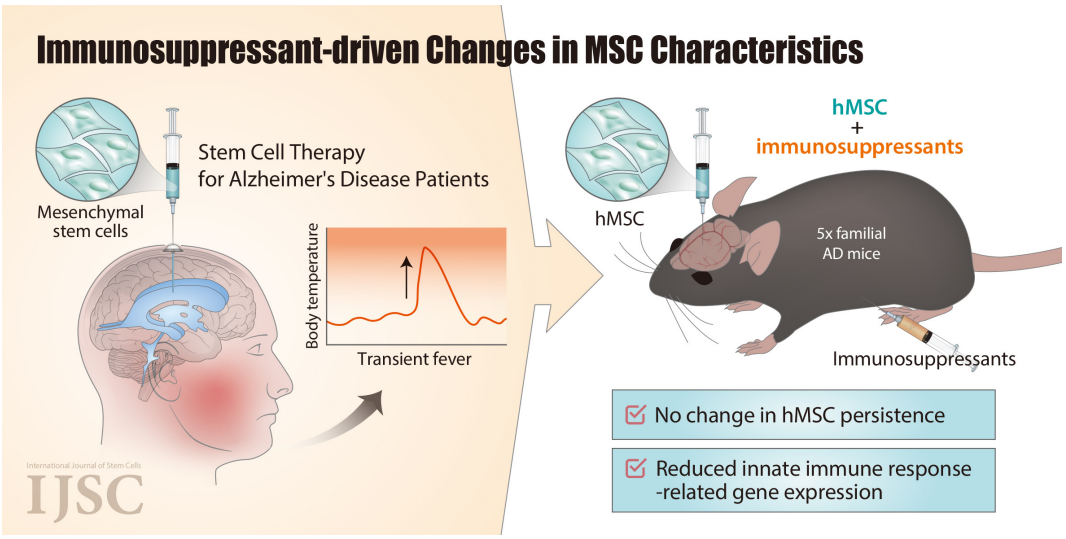

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Mesenchymal stem cells (MSCs) are frequently used for therapeutic applications in both pre-clinical and clinical settings owing to their capacity for immune modulation and neuroprotective effects. However, transient fever is commonly observed as an adverse event following MSC injection in patients with Alzheimer's disease (AD). In this study, we investigated the potential impact of immunosuppressants such as dexamethasone and tacrolimus on altering the characteristics of human mesenchymal stem cells (hMSCs). Additionally, we examined whether these immunosuppressants affect the persistence of hMSCs or the immune response upon their administration into the brain parenchyma of AD mice. The exposure of hMSCs to high concentrations of dexamethasone and tacrolimus *in vitro* did not significantly alter the characteristics of hMSCs. The expression of genes related to innate immune responses, such as *Irak1*, *Irf3*, *Nod1*, and *Ifnar1*, was significantly downregulated by the additional administration of dexamethasone and tacrolimus to the brain parenchyma of AD mice. However, hMSC persistence in the AD mouse brain was not affected. The results of this study support the use of immunosuppressants to mitigate fever during stem cell therapy in patients with AD.

Keywords: Alzheimer's disease, Immunosuppressive agents, Mesenchymal stem cells, Fever, Immunity

Introduction

Over the years, researchers have widely proposed and accepted the use of stem cells as a therapeutic option for a wide spectrum of diseases. Mesenchymal stem cells (MSCs) are frequently used in both preclinical and clinical settings because of their advantageous properties, including high accessibility, yield, paracrine activity, and

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hypo-immunogenicity (1, 2). MSCs secrete various cytokines and factors related to immune modulation and neuroprotection in diseased environments through their paracrine activity, which provides therapeutic benefits (3-5). The diverse MSC secretome has been shown to regulate both innate and adaptive immune cell populations via proteins such as prostaglandin E2, indoleamine 2,3-dioxygenase, and hepatocyte growth factor (6). Moreover, MSCs facilitate the repair of injured neurons by secreting neurotrophic factors such as neurotrophin-3, brain-derived neurotrophic factor, and nerve growth factor (6, 7).

Recently, the United States Food and Drug Administration approved two amyloid-beta-targeting drugs, lecanemab (8) and donanemab (9), to treat Alzheimer's disease (AD). However, it is important to note that multiple factors beyond amyloid contribute to the pathogenesis of AD. MSCs have been reported to reduce amyloid plaques (10) and tau (11), promote neuroprotective effects (12), and enhance proteasomal activity (13). Therefore, while only early stages of AD may benefit from amyloid-targeted therapy, patients with more advanced stages of AD may benefit from human mesenchymal stem cells (hMSCs).

In our previous phase I clinical trial, transient fever was observed as an adverse event following MSC injection in patients with AD (14). As patients exhibited discomfort, steroid administration was incorporated into the study design. A recent study reported that immunosuppressants improved MSC persistence in wild-type mouse parenchyma and reduced MSC-induced immune and inflammatory responses at the injection site (15). However, the potential of combining dexamethasone and tacrolimus in AD stem cell therapies has not been thoroughly examined, as it has not been tested in AD mouse models.

The major objective of this study was to examine whether dexamethasone and tacrolimus, either alone or in combination, alter the characteristics of hMSCs. Second, we assessed the effects of co-administration of dexamethasone and tacrolimus on the persistence of hMSCs injected into the parenchyma of 5x familial AD (5xFAD) mice. Furthermore, we investigated how these immunosuppressant regimens modulate immune responses exerted by injected hMSCs in the 5xFAD mice.

Materials and Methods

Evaluation of cell morphology and viability following treatment with varying concentrations of dexamethasone, tacrolimus, or both

In vitro concentrations of dexamethasone and tacrolimus were determined by referring to the clinical dosages of each immunosuppressant (16). When 10 mg of dexamethasone is administered intravenously in humans, the peak plasma concentration (Cmax) is 9.4±0.2 ng/mL, resulting in an expected cellular concentration of around 0.03 μM. Similarly, when 3 mg tacrolimus is administered orally in humans, the Cmax is 9.4±0.2 ng/mL, resulting in an expected cellular concentration of approximately 0.018 μM. The effects of dexamethasone and tacrolimus on MSCs were evaluated at these respective concentrations. MSCs were treated with different concentrations of dexamethasone only (0.005, 0.05, 0.5, 5, and 50 μM), tacrolimus only (0.005, 0.05, 0.5, 5, and 50 μM), and combinations of dexamethasone and tacrolimus (5 μM dexamethasone+5 μM tacrolimus and 50 μM dexamethasone+50 μM tacrolimus) for 24 hours.

Cell morphology was observed using an inverted microscope (ECLIPSE Ts2-FL Microscope; Nikon). Cell viability was analyzed using a cell counting kit-8 (CCK-8) (CK04; Dojindo) according to the manufacturer's instructions. Absorbance was read using a microplate reader (SpectraMax ABS Plus; Molecular Devices) at 450 nm. All samples were analyzed in triplicate.

Fluorescence-activated cell sorting analysis

To confirm the characteristics of MSCs, their surface antigen expression was analyzed using flow cytometry (BD FACS Aria III Flow Cytometer; BD Biosciences). Cells were harvested from the culture and centrifuged at 20,000 *g* for 3 minutes, and the cell density was adjusted to 1×10^6 cells/mL. The cells were resuspended in phosphate-buffered saline (PBS, 10010023; Gibco, Thermo Fisher Scientific) supplemented with 15% fetal bovine serum and treated with anti-CD44-FITC (560977; BD Biosciences), anti-CD73-APC (560847; BD Biosciences), anti-CD105-APC (562408; BD Biosciences), anti-CD166-PE (559263; BD Biosciences), anti-CD14-PE (555398; BD Biosciences), anti-CD19-FITC (555412; BD Biosciences), CD34-PE (555822; BD Biosciences), and anti-HLA-DR-PE (555812, mouse; BD Biosciences) antibodies. Surface marker expression was quantified using the MACSQuant Analyzer (Miltenyi Biotec).

Analysis of MSC tri-lineage differentiation potential

MSCs were seeded in 12-well plates (150628; Gibco, Thermo Fisher Scientific) to induce adipogenic differentiation at a density of 2.0×10^4 cells per well. The media was changed twice a week for three weeks using the StemPro Adipogenesis Differentiation Kit (A10070-01; Thermo Fisher Scientific). The cells were fixed in 4% paraformaldehyde for 1 hour, stained with Oil Red O (O0625; Sigma-Aldrich), and observed under a light microscope (Ts2-FL).

To induce osteogenic differentiation, MSCs were seeded (2.0×10^4 cells/well) into each well of a 12-well plate. The StemPro Osteogenesis Differentiation Kit (A10072-01; Thermo Fisher Scientific) was used to change the medium twice per week for four weeks. The cells were fixed in 4% formaldehyde (47608; Sigma-Aldrich) for 1 hour, stained using an Alizarin Red S staining kit (0223; ScienCell), and examined under a microscope (Ts2-FL) for positive staining.

MSCs (equivalent to 5.0×10^5) were cultured in chondrogenic induction media for four weeks to induce differentiation. Chondrogenic media included high-glucose Dulbecco's modified Eagle's medium (LM001-05; Welgene), 50 mg/mL L-ascorbic acid (A4403; Sigma-Aldrich), 100 ng/mL sodium pyruvate (P5280; Sigma-Aldrich), 40 mg/mL L-proline (P5607; Sigma-Aldrich), 100 μM dexamethasone (D4902; Sigma-Aldrich), 10 ng/mL transforming growth factor beta 3 (TGFβ-3, 8420-B3; R&D Systems), 500 ng/mL bone morphogenetic protein-6 (BMP-6, 507-BP; R&D Systems), and 50 mg/mL ITS$^+$ premix (354352; Corning). To confirm the differentiation process, cells were fixed using Tissue-Tek O.C.T. Compound (6200; Sakura Finetek Europe) and stained with 0.1% safranin O (477-73-6; Biosesang). Quantitative analysis of chondrogenic differentiation was performed using the ImageJ software (National Institutes of Health).

Ethical statement and experimental animals

This study protocol was reviewed and approved (approval number: 20210708001; date: July 8, 2021) by the Institutional Animal Care and Use Committee of the Research Institute for Future Medicine at Samsung Medical Center. All experiments were conducted as per the ARRIVE guidelines 2.0. The Institute adheres to the guidelines set by the Institute of Laboratory Animal Resources and is an accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care International. Genotyping was performed using genomic DNA (gDNA) extracted from the tail snips of littermates born by mating 5xFAD and C57BL6/J mice, originally purchased from the Jackson Laboratory. To be considered transgenic, mice had to express both the amyloid precursor protein and the presenilin-1 genes. Experiments were performed using transgenic AD mice that were 8～9 months old (total of 23 mice: 11 males, 12 females, average body weight 27 g). The mice were maintained in a 12 hours light/dark cycle and fed *ad libitum*.

MSC administration and co-administration of immunosuppressants

Human MSCs derived from Wharton's jelly, as previously reported (15-17), were used for this study (Samsung Medical Center, IRB No. 2016-07-102). On day 0, each mouse was injected with 2×10^5 MSCs suspended in 5 μL of minimal essential alpha 1x medium, without phenol red, into the left caudate putamen. The stereotaxic coordinates were anterior/posterior (A/P) −0.5 mm, medial/lateral (M/L) −1.7 mm, and dorsal/ventral (D/V) −3.3 mm. During stereotactic administration, mice were anaesthetized with 2% isoflurane (Hana Pharmaceuticals), and their heads were fastened using ear bars. Cells were injected at a rate of 1 μL/min, with a 5-minute delay prior to removing the Hamilton syringe to prevent backflow. Following the procedure, the skin incision was sutured, and before returning them to their cage, the mice were placed on a heating pad to facilitate full recovery. No specific randomization method was used for mouse or cage allocation. A subcutaneous injection of ketoprofen was provided if postoperative pain was observed. Mice were monitored weekly for any adverse effects, including weight loss, pallor, hair loss, and weakness.

The immunosuppressant regimen schedule is shown in Supplementary Fig. S1. Tacrolimus (Astellas Pharma) was administered intraperitoneally once daily (3 mg/kg), from day −1 up to the sacrifice time point on day 7, for both conditions 1 and 2. Dexamethasone (Jeil Pharmaceutical Co., Ltd.) was orally administered once a day (1 mg/kg) on days −1 and 0 for condition 1 or daily for condition 2. The doses of immunosuppressants were equivalent to those used in wild-type mice to study the effects of each immunosuppressant on the persistence and immune response of the administered MSCs (15). On the day of sacrifice (day 7), Mice were euthanized by cardiac perfusion (only PBS) under deep anesthesia. Brain tissue samples were harvested, and only the left hemisphere (including the cerebellum), the site of cell administration, was used for analysis. Mouse group allocation was known to all authors in each stage of the experiment.

Quantitative polymerase chain reaction analysis

To quantify residual human MSCs in the 5xFAD mouse brain parenchyma, the left hemisphere was homogenized, and gDNA was extracted from the following groups: hMSC (n=6) and hMSC+dexamethasone and tacrolimus (DexaTac) (n=6, condition 1; n=4, condition 2). For the hMSC+DexaTac (condition 1) group, one outlier was excluded according to the ROUT method (18). Real-time polymerase chain reaction (PCR) was performed by mixing the gDNA samples (final concentration: 500 ng) with Taq 2X probe master mix (final concentration: 1x; Promega), ALU primers (final concentration: 180 nm), and an ALU

probe (50 nM). The sequence (5'-3') of the ALU primers was as follows: forward, ACCTGAGGTCAGGAGTTTGAGA; reverse, ACCACGCCCGGCTAATTTT. The PCR conditions were as follows: initial denaturation, 95℃ for 10 minutes; denaturation, 95℃ for 15 seconds; annealing, 56℃ for 30 seconds; and extension, 72℃ for 30 seconds; total of 40 cycles. Six different standard samples, 5,000, 1,000, 200, 50, 8, and 1.6 pg/㎍ mouse DNA, were prepared to determine the concentration of human DNA in each respective sample (pg/㎍ mouse DNA).

PCR screening

Using the cDNA samples synthesized from RNA isolated from the left hemispheres of hMSC (n=3) and hMSC+DexaTac (n=4; condition 1) groups, the AccuTarget qPCR Screening Kit [Mouse-0089] [20X] Innate and Adaptive Immune Responses (Bioneer) was used to assess changes in relative expression of 64 genes associated with the mouse innate and adaptive immune response. Quantitative PCR (qPCR) conditions were as follows: step 1 (95℃, 10 minutes), step 2 (95℃, 5 seconds; 58℃, 25 seconds; 72℃, 30 seconds) for 40 cycles, step 3 (65℃, 5 minutes), and step 4 (melting curve analysis: 65℃～95℃, 1℃/s). For each group, if gene expression was not detectable in more than two biological replicates, the gene was excluded from the final analysis. Thus, out of the 64 genes, six in-

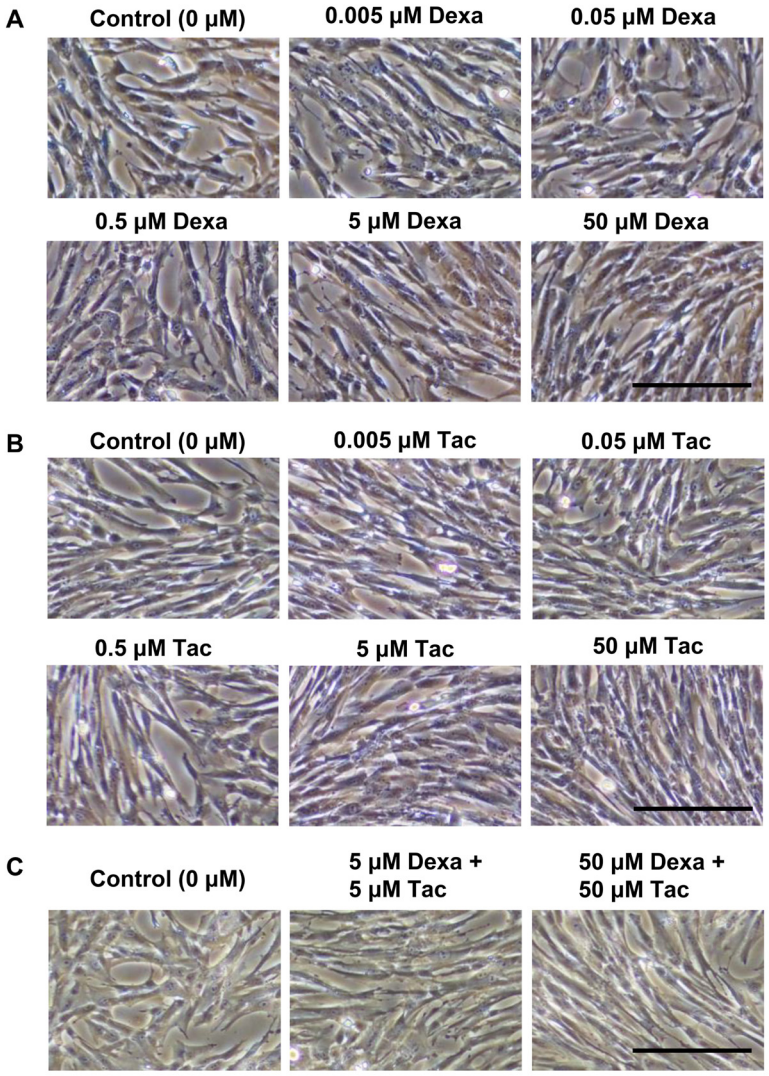

Fig. 1. Assessment of changes in the morphology of mesenchymal stem cells (MSCs) following treatment with dexamethasone (Dexa), tacrolimus (Tac), or combination treatment *in vitro* for 24 hours. Effects of exposure to various concentrations of immunosuppressant for 24 hours on the morphology of MSCs: Dexa (A), Tac (B), and both Dexa and Tac (C). MSC morphology was examined using an inverted light microscope. Scale bar=200 ㎛.

determinate genes were excluded, and the final analysis was performed on 58 genes. The $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen (19) was used to calculate changes in the relative expression of six different genes between the groups. The gene sequences were as follows: *Hsp90aa1*, forward: AAGCATAACGACGATGAGCA, reverse: CCTTTGT TCCACGACCCATT; *Hsf1*, forward: TCAAGCACAACAA CATGGCTA, reverse: CTGGAACTCGGTGTCATCTC; *Cryab*, forward: CTGGATTGACACCGGACTCT, reverse: CCTT GACTTTGAGTTCCTCCG; *Tnf-α*, forward: CACCACG CTCTTCTGTCTAC, reverse: GATGAGAGGGAGGCCA TTTG; *Il-6*, forward: TGTTCTCTGGGAAATCGTGG, reverse: CTGCAAGTGCATCATCGTTG; and *Il-1β*, forward: AAAGCTCTCCACCTCAATGG, reverse: TCGTTG CTTGGTTCTCCTTG.

Statistical analyses

GraphPad Prism 8.0 software was used for statistical analyses. Values are presented as the mean±SD. A p-value ≤0.05 was considered statistically significant. The CCK-8 assay and tri-lineage potential were analyzed using one-way ANOVA. The qPCR and PCR screening array results were analyzed using a t-test (unpaired, two-tailed).

Results

MSC morphology and viability following treatment with dexamethasone, tacrolimus, or both

To evaluate the effects of varying concentrations of immunosuppressants on the morphology and viability of MSCs, the cells were exposed to different concentrations of immunosuppressants for 24 hours. In the control (0 μM) group, MSCs maintained their traditional spindle-shaped and fibroblast-like appearance. As shown in Fig. 1A, MSCs treated with dexamethasone (0.005, 0.05, 0.5, 5, and 50 μM) exhibited similar morphology to that of the untreated control group. Similarly, MSCs treated with tacrolimus (0.005, 0.05, 0.5, 5, and 50 μM), also displayed morphology similar to the untreated control group (Fig. 1B). Additionally, when MSCs were treated with a combination of dexamethasone and tacrolimus (5 and 50 μM; Fig. 1C), there was no significant difference in cell morphology as compared to the control group.

Additionally, cell viability was examined using the CCK-8 assay, as shown in Fig. 2. The viability of MSCs treated with dexamethasone or tacrolimus (0.005, 0.05, 0.5, 5, and 50 μM) was not different from that of the untreated control group (Fig. 2A, 2B, respectively). Moreover, the viability of MSCs treated with the combination of dexamethasone and tacrolimus showed no significant difference between the untreated control group and the combined treatment group (5 and 50 μM; Fig. 2C).

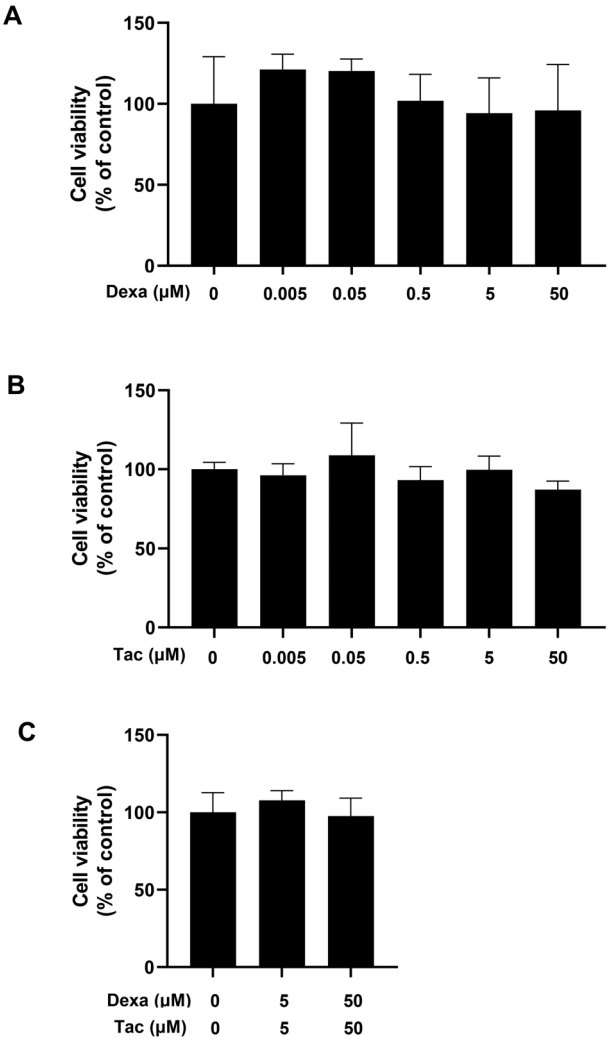

Fig. 2 Assessment of changes in viability of mesenchymal stem cells (MSCs) following treatment with dexamethasone (Dexa), tacrolimus (Tac), or a combination of both. Effects of different concentrations of Dexa (A), Tac (B), or a combination of Dexa and Tac (C) on cell viability. The viability of MSCs was measured using the CCK-8 assay. Values are expressed as mean±SD of three independent experiments. No significant difference was found between Dexa- and/or Tac-treated MSCs and untreated MSCs (control).

These results indicated that the morphology and viability of MSCs were not significantly altered after exposure to different concentrations of dexamethasone, tacrolimus, or a combination of both.

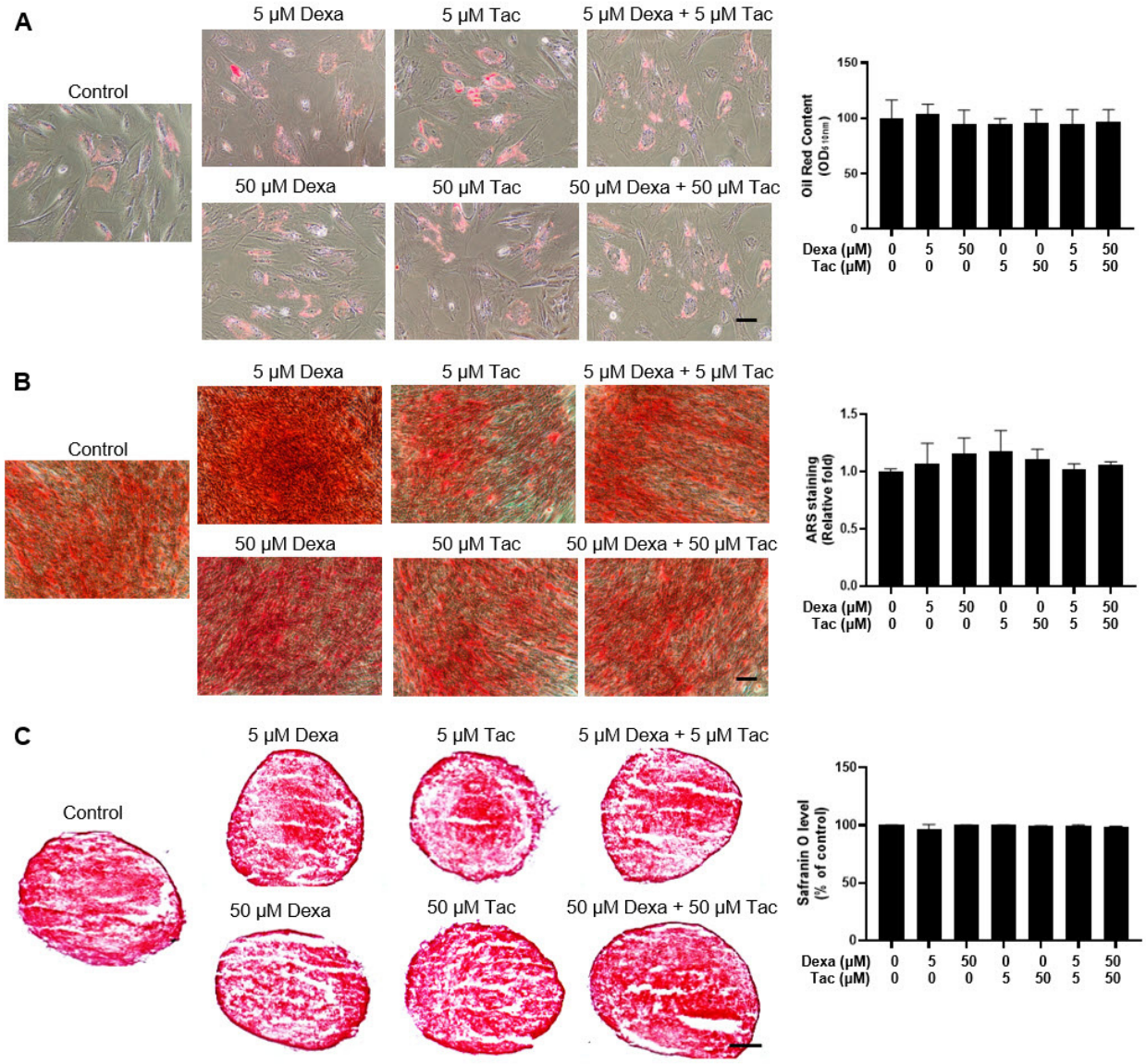

Fig. 3. Assessment of the tri-lineage differentiation potential of mesenchymal stem cells (MSCs) treated with dexamethasone (Dexa), tacrolimus (Tac), or a combination of Dexa and Tac. (A) Adipogenesis (Oil Red O staining), (B) osteogenesis (Alizarin Red S, ARS staining), and (C) chondrogenesis (safranin O staining) differentiation of MSCs treated with Dexa, Tac, or a combination of Dexa and Tac. Image J was used to quantify the tri-lineage potential of MSCs (% of control). Scale bar=200 μm. No significant difference was found in the Dexa- and/or Tac-treated MSCs compared to the untreated MSCs (control).

Tri-lineage differentiation of MSCs after treatment with immunosuppressants

We examined the tri-lineage differentiation ability of MSCs following immunosuppressant treatment to determine the identity of the MSCs. As shown in Fig. 3A, the adipogenic differentiation of MSCs treated with 5 and 50 μM of dexamethasone, tacrolimus, or the dexamethasone and tacrolimus combination was demonstrated by the presence of oil droplets on day 14, indicating ongoing adipogenesis. Similarly, the osteogenic differentiation of MSCs treated with 5 and 50 μM of dexamethasone, tacrolimus, or the dexamethasone and tacrolimus combination was indicated by the deposition of red-colored calcium phosphate, indicating ongoing osteogenesis (Fig. 3B). In addition, the chondrogenic differentiation of MSCs treated with 5 and 50 μM of dexamethasone, tacrolimus, or the dexamethasone and tacrolimus combination all exhibited positive safranin O staining, indicating ongoing chondrogenesis (Fig. 3C). These three quantitative analyses of tri-lineage differentiation indicate that MSC differ-

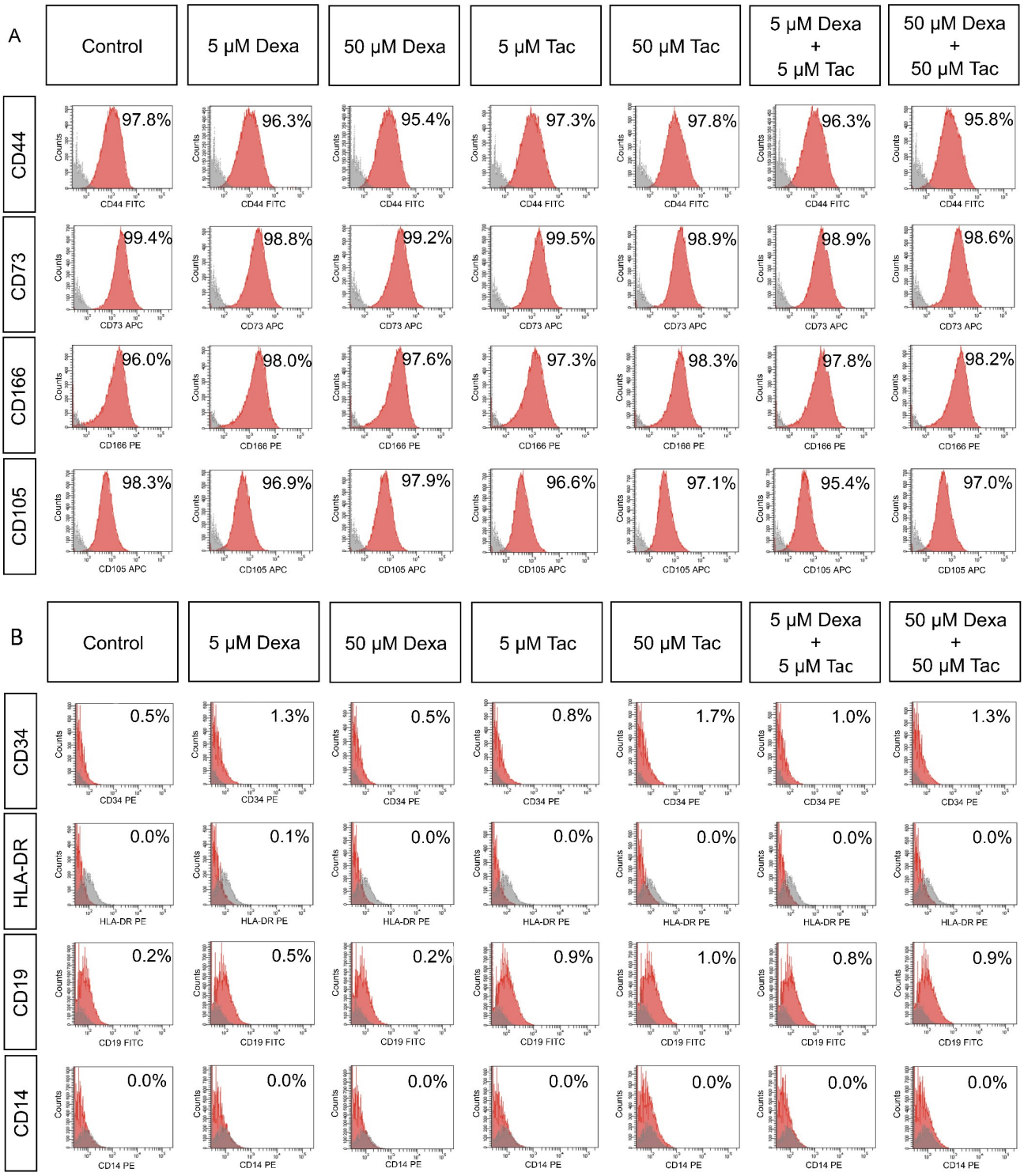

Fig. 4. Flow-cytometric analysis of mesenchymal stem cells (MSCs) following treatment with dexamethasone (Dexa), tacrolimus (Tac), or a combination of Dexa and Tac. (A) MSC surface markers, including CD44, CD73, CD166, and CD105, were highly expressed (>95%). (B) Hematopoietic markers, including CD34, HLA-DR, CD19, and CD14, were rarely expressed (<2%). The grey peaks correspond to the isotype control, while the red-coloured peaks correspond to the antibody of interest. FITC: fluorescein isothiocyanate, APC: allophycocyanin, PE: phycoerythrin.

entiation was sustained after immunosuppressant treatment.

Characterization of cluster of differentiation markers in MSCs after treatment with immunosuppressants

To determine changes in the expression of surface marker antigens after treatment with dexamethasone, tacrolimus, or a combination of both, the percentage of representative cluster of differentiation (CD) markers was quantified using fluorescence-activated cell sorting (Fig. 4). As per the International Society for Cellular Therapy (ISCT) criteria for MSCs (20), the expression of MSC-positive CD surface markers (CD44, CD73, CD166, and CD105) exceeded 95%, while the expression of MSC-negative CD surface markers (CD34, HLA-DR, CD19, and CD14) was below 2% for control MSCs and those treated with 5 and 50 μM of dexamethasone, tacrolimus, or a combination of both (Fig. 4).

Effect of dexamethasone and tacrolimus co-administration on MSC persistence

In the parenchyma of 5xFAD mice, the persistence of hMSCs exhibited no significant difference between mice receiving injections of pure hMSCs and those that were co-administered with dexamethasone and tacrolimus (Fig. 5). Between the two immunosuppressant regimens, condition 1 showed a higher fold increase (~1.7-fold) in MSC persistence compared to that of the hMSC-only group. Therefore, condition 1 was selected for further investigation of the changes in the immune response following the co-administration of dexamethasone and tacrolimus in hMSC-injected 5xFAD mice.

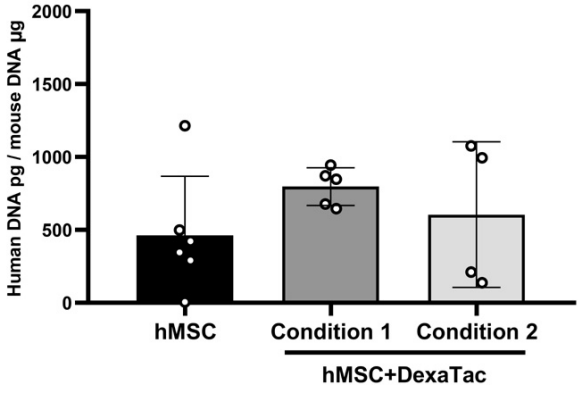

Fig. 5. Co-administration of Dexamethasone and Tacrolimus (DexaTac) does not significantly enhance human mesenchymal stem cell (hMSC) persistence in the 5xFAD parenchyma. Residual hMSCs were quantitated by dividing the human DNA concentration (pg) by the mouse DNA concentration (μg) in each group: hMSCs (n=6), condition 1 (n=5), and condition 2 (n=4). Although increased hMSC persistence was observed in each condition compared to the hMSC group, the differences were non-significant.

Effect of dexamethasone and tacrolimus co-administration on MSC-immune response exerted by MSCs

Fifty-eight genes related to mouse innate and adaptive immune pathways were included in the final analysis (Supplementary Table S1). Of the 58 genes, significant downregulation in the expression of four genes was observed in the hMSC+DexaTac group compared to that in the hMSC group: *Irak1* (fold change=0.23, p=0.001), *Ifnar1* (fold change=0.67, p=0.028), *Nod1* (fold change=0.64, p=0.035), and *Irf3* (fold change=0.74, p=0.038; Fig. 6A). No gene was significantly upregulated in the hMSC+DexaTac group compared to the hMSC group. Although not statistically significant, upregulation of interleukin-10 (IL-10) was observed in the hMSC group (fold change=2.5) compared to levels observed in the hMSC+DexaTac group.

The relative expression of heat shock protein (*Hsp90aa1*, *Hsf1*, and *Cryab*) and pro-inflammatory cytokine (*Tnf-α*, *Il-6*, and *Il-1β*) genes was quantified. All three heat shock genes were significantly downregulated in the hMSC+DexaTac group compared with their expression in the hMSC group: *Hsp90aa1* (fold change=0.57, p=0.026), *Hsf1* (fold change=0.58, p=0.013), and *Cryab* (fold change=0.74, p=0.040; Fig. 6B). Regarding pro-inflammatory cytokines, the hMSC+Dexa group showed significant downregulation of *Tnf-α* gene expression (fold change=0.59, p=0.009) compared with that in the MSC group, while no significant changes were observed for *Il-6* (p=0.517) and *Il-1β* (p=0.778; Fig. 6C).

Discussion

In this study, we investigated the effects of immunosuppressants on hMSCs in the context of AD treatment. *In vitro*, combined treatment with dexamethasone and tacrolimus did not alter the characteristics of hMSCs isolated from Wharton's jelly. Additionally, *in vivo*, the persistence of hMSCs in the brain parenchyma of 5xFAD mice was not altered when the immunosuppressants were administered. However, these immunosuppressants mitigated the immune response triggered by hMSCs in the brain. Taken together, our results provide evidence supporting the use of immunosuppressants to mitigate immune responses during stem cell therapy in patients with AD.

To determine whether immunosuppressants affect MSC properties, we characterized MSCs based on their morphology, viability, tri-lineage differentiation potential, and

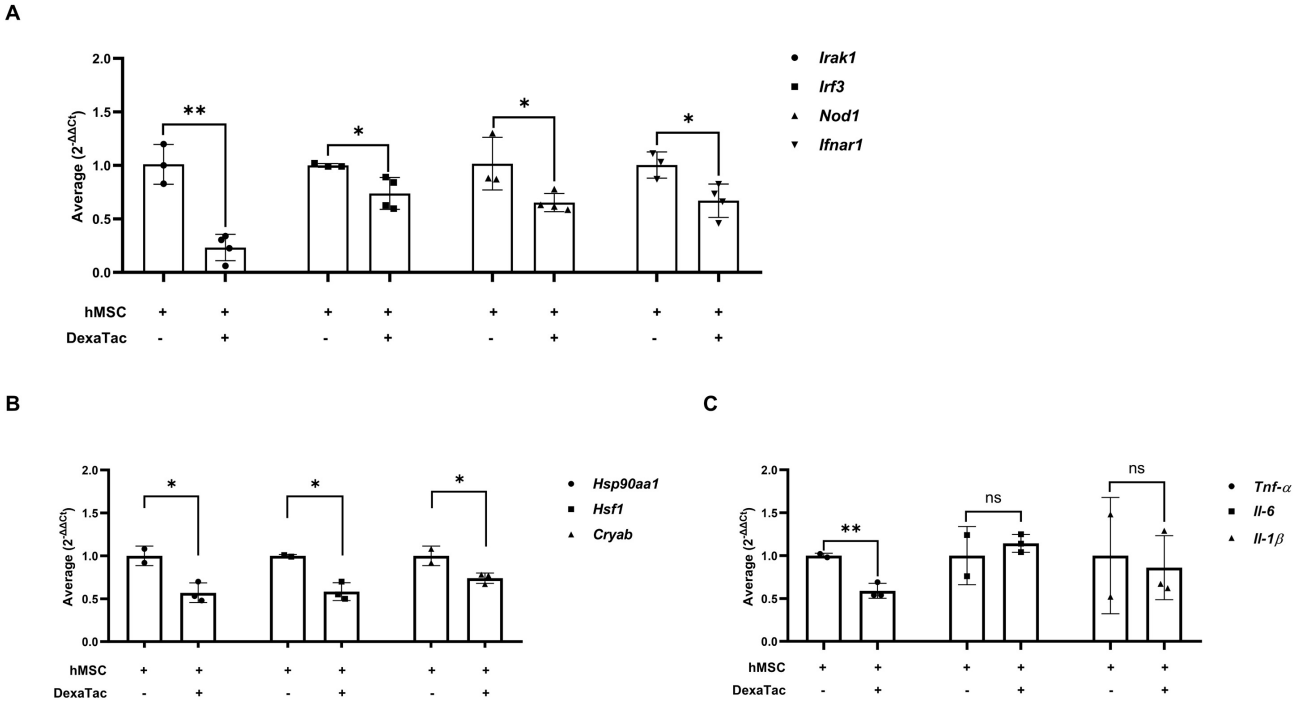

Fig. 6. Genes significantly downregulated following co-administration of Dexamethasone and Tacrolimus (DexaTac). (A) The average $2^{-\Delta\Delta Ct}$ values for each of the experimental animals in the human mesenchymal stem cell (hMSC) (n=3) and hMSC+DexaTac (n=4) groups are graphically illustrated. A statistically significant reduction in the expression of *Irak1, Irf3, Nod1,* and *Ifnar1* was observed when DexaTac were co-administered in hMSC-transplanted 5xFAD mice. (B) A statistically significant reduction in the expression of heat shock proteins, *Hsp90aa1, Hsf1*, and *Cryab* was observed when DexaTac were co-administered in hMSC-transplanted 5xFAD mice. (C) Gene expressions of pro-inflammatory cytokines, *Tnf-α, Il-6,* and *Il-1β* were also measured between the 2 groups. Only *Tnf-α* showed statistically significant reduction following co-administration of DexaTac. Statistical significance is defined as *p<0.05, **p<0.01 vs. hMSC; t-test, two-tailed, unpaired. Values are expressed as mean±SD. ns: not significant.

surface marker expression using ISCT criteria (16). Our findings indicate that MSCs treated with immunosuppressants maintained their stem cell characteristics without undergoing morphological changes or cell death. Furthermore, the stem cell characteristics remained intact as MSCs treated with immunosuppressants differentiated into various cell types, including adipocytes, osteoblasts, and chondrocytes, and expressed distinct stem cell surface markers. Therefore, these results suggest that treatment with immunosuppressants such as dexamethasone and/or tacrolimus, did not alter the properties of MSCs *in vitro*. While previous studies (21, 22) reported that dexamethasone promoted apoptosis and inhibited osteogenesis in MSCs, our findings demonstrated no significant effect on MSC viability or differentiation. This discrepancy may reflect differences in dosage, exposure duration, or experimental conditions. Importantly, in contrast to prior studies that focused on osteogenesis or apoptosis *in vitro*, our study investigated the role of immunosuppressants in the context of AD, exploring their effect on MSC persistence and immune modulation *in vivo*. By utilizing the 5xFAD mouse model, we provide insights into MSC therapy for neurodegenerative diseases, highlighting immune-related gene responses in brain parenchyma. Furthermore, we examined the combined effect of dexamethasone and tacrolimus, informing potential clinical protocols to mitigate adverse immune responses, which further distinguishes our work from previous single-agent studies. Consistent with *in vitro* findings, *in vivo* experiments showed that the co-administration of dexamethasone and tacrolimus did not significantly alter the persistence of hMSCs in the parenchyma of 5xFAD mice after 7 days. These results indicated that the proposed immunosuppressive regimen was not toxic and did not affect the viability of hMSCs injected into the parenchyma of 5xFAD mice. One animal in the group receiving only MSC demonstrated higher levels of MSC persistence compared with that in the other animals, a factor which may have been responsible for the

lack of statistical significance observed. This finding underscores the importance of increasing the sample size to reduce the impact of individual differences.

MSCs have been studied as a regenerative treatment for various neurodegenerative diseases. Several studies have highlighted the beneficial effects of MSCs in both animal models (23, 24) and patients (14, 25). Despite MSCs' immune-privileged characteristics, there have been instances of leukocyte infiltration and fever following transplantation into the central nervous system (14). Other studies have reported that MSCs are not immune-privileged or hypo-immunogenic; rather, they possess immune-evasive characteristics (26). Hwang et al. (27) reported that MSCs are immunogenic and may cause immunological issues. Administering immunosuppressants can reduce the immune response following stem cell transplantation and decrease the expression of immune cells at the site of MSC transplantation (15, 16).

Our results showed that immunosuppressants downregulated immune activity triggered by hMSCs in the brain. We quantified changes in the relative expression of numerous genes related to innate and adaptive immunity using brain tissue samples acquired from 5xFAD mice that received hMSCs with or without immunosuppressant administration. The co-administration of dexamethasone and tacrolimus resulted in a statistically significant downregulation of four genes (*Irak1*, *Irf3*, *Nod1*, and *Ifnar1*) compared to their expression in the group that received only hMSC injections. The critical role of *Irak1* in mediating inflammation and innate immunity is well established (28, 29). Notably, among the four genes, *Irak1* showed the lowest expression when dexamethasone and tacrolimus were administered with hMSCs. The interferon regulatory factor 3 (*Irf3*) is known to play a significant role in the production of pro-inflammatory cytokines related to innate immunity (30, 31). However, the role of *Irf3* in stem cell therapy remains poorly understood. The type 1 interferon (IFN) receptor consists of two subunits, interferon alpha and beta receptor subunit 1 (IFNAR1) and IFNAR2 (32), that play a crucial role in regulating innate and adaptive immune responses (33). The downregulation of IFNAR1 maintains the function of hematopoietic stem cells (34). Moreover, the results of this study demonstrate the therapeutic benefits of this gene in stem cell therapy. Notably, there was an upregulation of the expression of IL-10 following the co-administration of dexamethasone and tacrolimus in hMSC-treated 5xFAD mice, although the difference was not statistically significant. IL-10 is a widely recognized potent anti-inflammatory cytokine (35). The increase in IL-10 expression by more than two-fold suggests that the immunosuppressant regimen effectively addressed the immune responses caused by the injected hMSCs.

As previously reported, the intracerebroventricular injection of hMSCs in 5xFAD mice resulted in elevated pro-inflammatory cytokine levels, including TNF-α, IL-1β, and IL-6, in brain tissue lysates (36). These cytokines are known as pyrogenic or fever-related cytokines. Furthermore, studies have identified a correlation between *Irak1*, *Irf3*, *Nod1*, and *Ifnar1* and the production of fever-related inflammatory cytokines such as TNF-α and IL-1β (37). Given the potential association of IRAK1, IRF3, NOD1, and IFNAR1 with the pyrogenic-related cytokines TNF-α, IL-6, and IL-1β, we observed a significant decrease in *Tnf-α* expression following the co-administration of dexamethasone and tacrolimus. The lack of observed trends for *Il-6* and *Il-1β* may be attributed to the limited sample size and the differing sacrifice time points from the previous report, which observed elevated IL-6 and IL-1β levels following intracerebroventricular delivery of human MSCs in 5xFAD mice. In addition to that of *Tnf-α*, *Il-6*, and *Il-1β*, the expression of *Hsp90aa1*, *Hsf1*, and *Cryab* was evaluated. Heat shock proteins demonstrate increased expression in response to heat stress, including the induction of fever (38). The administration of DexaTac resulted in a notable downregulation of all three genes (*Hsp90aa1*, *Hsf1*, and *Cryab*) in 5xFAD mice. These findings indicate that DexaTac may have played a role in reducing fever and potentially influencing body temperature.

Our study had several limitations. First, the small sample size may have contributed to the lack of statistically significant results for ALU qPCR findings. It is possible that more than four genes, including *Il-10*, would show statistically significant differences between the hMSC-only and hMSC+DexaTac groups with a larger sample size. Therefore, future studies should consider increasing the sample size. Second, although many genes were quantified, the results of the PCR screening array were not validated by other methods or assays, such as immunohistochemistry or enzyme-linked immunosorbent assay. Third, the body temperature of the experimental animals was not monitored. Nevertheless, body temperature was indirectly assessed through the measurement of the gene expression of representative heat shock proteins. However, the absence of direct body temperature measurements and systemic cytokine profiling limits the ability to evaluate systemic hyperinflammation, which may have contributed to fever in AD patients receiving MSC therapy. The 5xFAD model predominantly reflects localized neuroinflammation and does not fully replicate the systemic immune dysregu-

lation observed in clinical settings. Consequently, it is imperative that future studies encompass the analysis of peripheral blood cytokines to provide a more comprehensive assessment of systemic inflammatory responses following MSC administration. Fourth, we were not able to assess the influence of immunosuppressants on the proteomic, transcriptomic, and secretomic profiles of hMSCs. Finally, we did not evaluate the long-term safety and efficacy of co-administration of DexaTac with hMSC injection.

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Potential Conflict of Interest

Jong Wook Chang is currently employed by ENCell Co., Ltd. ENCell Co., Ltd was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication. All the other authors have no potential conflict of interest to declare.

Availability of Data and Materials

All the data used to support the findings of this study are included within the article.

Authors' Contribution

Conceptualization: NKL, DLN, HJS, HJK. Data curation: NKL, NHL, HJS. Formal analysis: NKL, DLN, HJS. Funding acquisition: NKL, HJS. Investigation: NKL, HJS. Methodology: NKL, SHM, SYL, HJS. Project administration: NKL, HJS, HJK. Resources: SWS, JWC. Validation: NKL, HJS, HJK. Writing-original draft: NKL, HJS, DLN, HJ, HJK. Writing – review and editing: NKL, HJS, HJK.

Supplementary Materials

Supplementary data including one table and one figure can be found with this article online at https://doi.org/10.15283/ijsc24116

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