

Tumor necrosis factor- α -treated human adipose-derived stem cells enhance inherent radiation tolerance and alleviate *in vivo* radiation-induced capsular contracture

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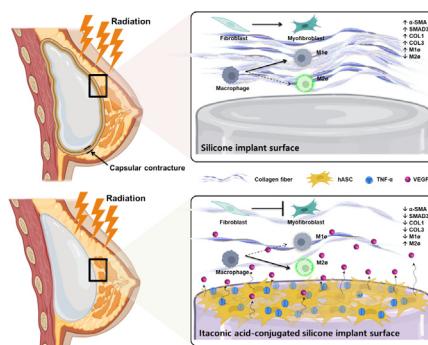
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HIGHLIGHTS

- Hydrophilic modification with itaconic acid improves PDMS biocompatibility and ADSC attachment *in vitro*.
- TNF- α treatment enhances inherent radiation tolerance in human ADSCs.
- TNF- α -treated PDMS and IA-PDMS implants reduce fibrosis factors *in vivo* and promote M2 macrophage polarization.
- TNF- α -treated human ADSCs release more VEGF, aiding immune response modulation and mitigating radiation-induced capsular contracture.
- TNF- α -treated human ADSCs emerge as a promising strategy to alleviate post-mastectomy and radiation therapy complications in breast reconstruction.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Post-mastectomy radiotherapy plays a crucial role in breast cancer treatment but can lead to an inflammatory response causing soft tissue damage, particularly radiation-induced capsular contracture (RICC), impacting breast reconstruction outcomes. Adipose-derived stem cells (ADSCs), known for their regenerative potential via paracrine capacity, exhibit inherent radiotolerance. The influence of tumor necrosis factor- α (TNF- α) on ADSCs has been reported to enhance the paracrine effect of ADSCs, promoting wound healing by modulating inflammatory responses.

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Objective: This study investigates the potential of TNF- α -treated human ADSCs (T-hASCs) on silicone implants to alleviate RICC, hypothesizing to enhance suppressive effects on RICC by modulating inflammatory responses in a radiation-exposed environment.

Methods: *In vitro*, T-hASCs were cultured on various surfaces to assess viability after exposure to radiation up to 20 Gy. *In vivo*, T-hASC and non-TNF- α -treated hASC (C-hASCs)-coated membranes were implanted in mice before radiation exposure, and an evaluation of the RICC mitigation took place 4 and 8 weeks after implantation. In addition, the growth factors released from T-hASCs were assessed.

Results: *In vitro*, hASCs displayed significant radiotolerance, maintaining consistent viability after exposure to 10 Gy. TNF- α treatment further enhanced radiation tolerance, as evidenced by significantly higher viability than C-hASCs at 20 Gy. *In vivo*, T-hASC-coated implants effectively suppressed RICC, reducing capsule thickness. T-hASCs exhibited remarkable modulation of the inflammatory response, suppressing M1 macrophage polarization while enhancing M2 polarization. The elevated secretion of vascular endothelial growth factor from T-hASCs is believed to induce macrophage polarization, potentially reducing RICC.

Conclusion: This study establishes T-hASCs as a promising strategy for ameliorating the adverse effects experienced by breast reconstruction patients after mastectomy and radiation therapy. The observed radiotolerance, anti-fibrotic effects, and immune modulation suggest the possibility of enhancing patient outcomes and quality of life. Further research and clinical trials are warranted for broader clinical uses.

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Introduction

Breast reconstruction is a crucial aspect of post-mastectomy care for breast cancer patients, frequently involving the utilization of silicone implants, even when radiation therapy is planned [1]. Despite the success of implant-based reconstruction after radiation, concerns remain about the negative effects of radiation, specifically radiation-induced capsular contracture (RICC) [2–6]. RICC is caused by a complex interplay of inflammatory responses and oxidative stress triggered by dysregulated matrix metalloproteinases (MMPs) following radiation injury, leading to abnormal extracellular matrix (ECM) accumulation [7,8]. Therefore, effective management of RICC risk is critical for optimal breast reconstruction outcomes.

Although there are several strategies to mitigate capsular contracture, interventions specifically targeting RICC remain relatively underexplored [9,10]. Woo et al. have highlighted the prophylactic potential of acellular dermal matrix (ADM) and montelukast against RICC [11]. However, these approaches have limitations and may pose systemic complications, including financial burdens [12,13]. Therefore, exploring alternative cost-effective and productive approaches to address RICC is imperative.

Recently, adipose-derived mesenchymal stem cells (ADSCs) have emerged as promising candidates in regenerative medicine because of their cost-effectiveness, ease of isolation, and minimal ethical concerns [14–17]. The autologous provision of ADSCs from abundant adipose tissue circumvents immune rejection, making them a safe option [18]. Moreover, their ability to differentiate into various cell types, release trophic factors, and modulate immune responses [15,19,20] renders them ideal for tissue repair and regeneration. Studies suggest that autologous fat grafting involving ADSCs has the potential to improve skin compliance in post-radiation fibrosis [21–23].

Beyond their regenerative capabilities, ADSCs exert crucial immunoregulatory effects via paracrine signaling, displaying immunosuppressive effects on immune cells and protecting tissues against ischemia-reperfusion injury [24,25]. Numerous studies have explored the utilization of ADSCs to mitigate fibrosis and capsular contracture [26,27]. Building on this foundation, our previous studies have demonstrated the potential of itaconic acid (IA) conjugation on polydimethylsiloxane (PDMS) membranes (IA-PDMS) to enhance biocompatibility *in vitro* and reduce capsular contracture *in vivo* [28,29]. Furthermore, our evaluation of human ADSCs (hASCs) cultured on PDMS and IA-PDMS membranes revealed a

significant reduction in capsular contracture compared to membranes without hASCs [30]. Recent findings indicate that ADSCs are inherently radioresistant [31,32], allowing them to withstand radiation damage and maintain their regenerative capabilities. Further exploration of the therapeutic effects of ADSCs in radiation-induced tissue injury holds significant value for improving breast reconstruction in patients undergoing radiation treatment.

Given the importance of modifying biological factors to enhance the therapeutic potential of ADSCs, pre-treatment strategies involving exposure to pro-inflammatory mediators have gained traction [33–35]. Among these mediators, tumor necrosis factor- α (TNF- α) emerges as a promising candidate because it modulates inflammation and promotes tissue repair processes [36,37]. A study by Prockop et al. demonstrated that TNF- α can activate mesenchymal stem cells (MSCs) to secrete anti-inflammatory proteins, thereby attenuating excessive inflammatory responses [38]. Furthermore, it has been demonstrated that TNF- α treatment can enhance the therapeutic potential of ADSCs by promoting the secretion of various factors conducive to wound healing and angiogenesis [39,40]. In addition, TNF- α has been reported to facilitate the migration of MSCs towards chemokines [41] and enhance invasive capacity by upregulating the expression of MMPs [42]. Considering the dysregulated MMP activity implicated in RICC [7,8], TNF- α -induced upregulation of MMPs may hold promise in mitigating RICC severity by facilitating tissue repair processes. Despite limited research on TNF- α signaling in ADSC-based therapy for immune response-related RICC, the radiation-resistant capacity of ADSCs suggests promising avenues for investigation. We hypothesize that incorporating TNF- α treatment into hASC-coated implants may suppress RICC via its paracrine effect and modulate inflammatory responses.

This study attempts to provide valuable insights into regenerative medicine and breast cancer treatment complications, aiming to enhance breast reconstruction outcomes and patient satisfaction.

Materials and methods

Fabrication and characterization of silicone membranes

Polydimethylsiloxane (PDMS) and IA-PDMS silicone membranes were fabricated via the methods outlined in previous studies [26]. Briefly, to obtain PDMS membranes, the curing agent and base reagent of the Sylgard® 184 Silicone Elastomer Kit (Dow Corn-

ing, Midland, MI) were mixed in a ratio of 1 part to 10 parts (w/w) and subsequently punched into disks (diameter, 1.0 cm). The IA-PDMS membrane was obtained by treating the PDMS surface with oxygen plasma (100 W, 5×10^{-2} torr, 1 min; CUTE-1B, Femto Science, Hwaseong, Korea). The membrane was subsequently incubated in 5 wt% (3-aminopropyl)triethoxysilane (Sigma-Aldrich, St. Louis, MO) at 60 °C for 2 h, followed by another incubation in a 150-mM IA solution containing 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich) and 50mM *N*-hydroxysuccinimide (Sigma-Aldrich) at 60 °C for 2 h. All membranes were sterilized with ethylene oxide gas at 38 °C for 4 h (SE30, ALOPS Corp., Gunpo, Korea). Surface hydrophilicity was determined via contact angle analysis (Phoenix-MT, Suwon, Korea), and chemical bonds were identified via attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR; Nicolet 6700, Thermo Fisher Scientific, Waltham, MA).

Isolation of hASCs and cell culture

Human ADSCs were isolated from healthy female patients who underwent liposuction, had no inflammation or cancer, and provided informed consent. Briefly, the obtained lipoaspirate was washed with Dulbecco's phosphate-buffered saline (DPBS; modified 1x, pH 7.4; HyClone Laboratories, Logan, UT) supplemented with a 1 % antibiotic/antimycotic solution (HyClone). The enzymatic breakdown was done with 0.01 % collagenase type I (Sigma-Aldrich) in a shaking water bath at 37 °C for 3 h. The cells underwent centrifugation at 1,300 rpm for 3 min, generating the stromal vascular fraction (SVF). The SVF was then reconstituted in a complete culture medium comprising 10 % fetal bovine serum (HyClone) and 1 % antibiotic/antimycotic solution in Dulbecco's modified Eagle's medium low-glucose (HyClone). The resuspended cells were filtered through a 100- μ m cell strainer (BD Biosciences, Bedford, MA). Subsequently, the cells were moved to a cell culture dish, cultured in complete media, and placed in a humidified incubator (HERAEUS BB 15; Thermo Fisher Scientific, Seoul, Korea) at 37 °C with 5 % CO₂. The complete medium was changed every 48 h, and the cells were washed twice with DPBS before utilization in subsequent procedures. Here, hASCs at Passages 3–6 were utilized.

Characterization of hASCs

Characterization of hASCs involved assessing the expression of surface markers such as CD45, CD90, CD73, and CD105. The hASCs were harvested from a monolayer culture dish utilizing a 1x solution of 0.25 % trypsin (HyClone). A suspension of individual cells was created and treated with specific antibodies, including FITC-conjugated CD45, PE-conjugated CD73, CD105, and PerCP-conjugated CD90 (BD Pharmingen, San Jose, CA). Flow cytometry was utilized to analyze the samples with a BD FACS Aria II machine (Becton Dickinson, NJ), and the data obtained were analyzed with FlowJo software (Treestar, Woodburn, OR).

Treatment of hASCs with TNF- α

The hASCs were seeded at 2.5×10^4 cells on culture plates and silicone membranes (PDMS and IA-PDMS) surfaces in 48-well plates. Upon reaching 80 % confluency (on Day 3), a complete medium containing 25 ng/mL TNF- α (PeproTech, Inc., Seoul, Republic of Korea) was utilized to culture the hASCs, designated as T-hASCs. Cells cultured without TNF- α served as the control group. For *in vivo* experiments, the cells were further cultured for 48 h before being evaluated for cell number viability and utilized *in vivo*.

Assessment of cell cytotoxicity, viability, and adhesion

Cell cytotoxicity and proliferation were evaluated with the CCK-8 assay. CCK-8 solution (10 % in a complete medium; Dojindo, Tokyo, Japan) was added to culture plates, and the plates were incubated at 37 °C for 2 h. The absorbance at 450 nm was quantified with a microplate reader (Bio-Tek ELx-800; BioTek, Winooski, VT, USA). The cell amount was calculated based on a standard curve (Fig. S1).

Cell viability and adhesion were assessed following the manufacturer's instruction utilizing the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific). Stained cells were observed under fluorescence microscopy (OX.2053-PLPH; Euromax, Roermond, Netherlands).

Investigation of cell cytoskeleton

After seeding the hASCs (1×10^4 cells) on culture slides for 24 h, the cells were washed twice with DPBS and further cultured with or without 25 ng/mL TNF- α in a complete medium for 48 h. Following this, the cultured cells were fixed with 4 % paraformaldehyde (FUJIFILM Wako Pure Chemical, Osaka, Japan) for 20 min and permeabilized with 0.3 % Triton X-100 (Sigma-Aldrich) in PBS for 10 min. Subsequently, the cells were incubated in 5 % BSA (Sigma) in PBS to block non-specific protein binding and then stained with an Alexa Fluor™ 488-conjugated vinculin monoclonal antibody (1:100, Invitrogen, Carlsbad, CA) at 4 °C overnight. A second staining was conducted with rhodamine-phalloidin (Thermo Fisher) at 25 °C for 2 h. Following this, the cells were further stained with DAPI (Thermo Fisher) for 3 min. DPBS washes were performed three times between each step. Finally, the stained cells were examined under fluorescence microscopy with a DM750 model (Leica Microsystems, Wetzlar, Germany).

Simulation and radiation planning

Before irradiation, simulation and radiation planning were conducted to ensure accurate targeting of the radiation dose to the implant, as illustrated in Fig. S2. Mice underwent computed tomography (CT) scanning utilizing a Philips Brilliance Big Bore 16-slice CT scanner with a slice thickness of 1 mm. The CT datasets were then transferred to the Eclipse treatment planning system (Version 10), where the implant was delineated, and the radiation plan was developed. An electron block was utilized to protect the head and neck of the mice from irradiation and exclude these areas from the radiation field. The radiation plan was meticulously verified to confirm that the planned dose was delivered precisely to the implant while ensuring that the head and neck regions remained unexposed to radiation.

Irradiation

Irradiation was conducted with a Varian 21EX Linear Accelerator (LINAC; Varian Medical Systems, Palo Alto, CA) at a dosage rate of 300 cGy/min with 6 MeV electron beams. After 48 h of TNF- α treatment, cultured cells were exposed to a single radiation dose of either 10 or 20 Gy. For *in vivo* experiments, the RICC mouse model was performed according to the methods described by Katzel et al. and Kim et al. [43,44]. The mice received radiation one week after implantation while anesthetized by administering ketamine (10 mg/kg Ketalar; Yuhan Corporation, Seoul, Korea) and xylazine (2.5 mg/kg Rompun; Bayer Corp., Whippany, NJ) via intraperitoneal injection. The diameter of the irradiation field was 25 cm, and three mice were irradiated simultaneously. The mice received localized irradiation on their dorsal areas with a single dose of 10 Gy.

Quantitative evaluation of released cytokines and growth factor

Semi-quantification of the released growth factor from the cells was conducted utilizing the Human Growth Factor Array C1 and Human Cytokine Array C5 (RayBiotech, Peachtree Corners, GA) in accordance with the manufacturer's guidelines. The chemiluminescent pictures were taken with the ImageQuant™ LAS 4000 imager (GE Healthcare, Chicago). The array images were evaluated with ImageJ software (ver. 1.47, National Institutes of Health, Rockville, MD). VEGF levels in the culture media were quantified with a Human VEGF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm. The concentration of human VEGF in the collected medium was determined by reference to a standard curve (Fig. S3).

Animal experiments

Eight 8-week-old female BALB/c mice weighing between 20 and 25 g were employed to assess the effect of C-hASCs and T-hASCs on capsular contracture around the implants. In addition, another four 8-week-old female BALB/c mice within the same weight range were utilized to evaluate the impact of irradiation on apoptosis in the local tissue surrounding the implants. The mice were kept in a specific pathogen-free environment with a 12-h light/12-h dark cycle and were given unrestricted access to water and food. To process silicone implantation, their dorsal area was entirely shaved to eliminate hair, and incisions (1.0–1.5 cm) were made on the shaved dorsal region with surgical scissors. Subcutaneous

pockets were created through the incisions, and four pieces of the implant samples were inserted, as illustrated in Fig. 1A and B. After washing twice with DPBS, C-hASC on PDMS (C-PDMS) was inserted at Position 1 (control); C-hASC on IA-PDMS (C-IA-PDMS) at Position 2; T-hASC on PDMS (T-PDMS) at Position 3; and T-hASC on IA-PDMS (T-IA-PDMS) at Position 4. Nylon 4/0 sutures (ETHILON, New Brunswick, NJ) were utilized for incision closure, and a betadine solution was applied to disinfect the area, preventing external stimulation and infection. Biopsies were performed, and mice were euthanized with CO₂ at 24 h after radiation, as well as at 4 and 8 weeks after implantation (n = 4 for each time point). Tissues were immediately stored at -80 °C for quantitative analysis. To conduct *in vivo* staining, tissues were fixed in a 4 % formalin solution. Paraffin blocks were prepared from the biopsy tissues, and 4-μm-thick slices were stained for each factor.

Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR)

Total RNA was purified from the biopsy tissue with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with the AccuPower® Rocket-Script™ RT-PCR PreMix & Master Mix (Bioneer, Daejeon, Korea) to create cDNA from RNA (1 μg). The generated cDNA was stored at -20 °C until further evaluation. Quantitative PCR was conducted with the PowerSYBR Green PCR Master Mix (Thermo Fisher) on a StepOnePlus Real-Time PCR system (AB Applied, Life Technologies, MA). The control group ratio was utilized to standardize the gene

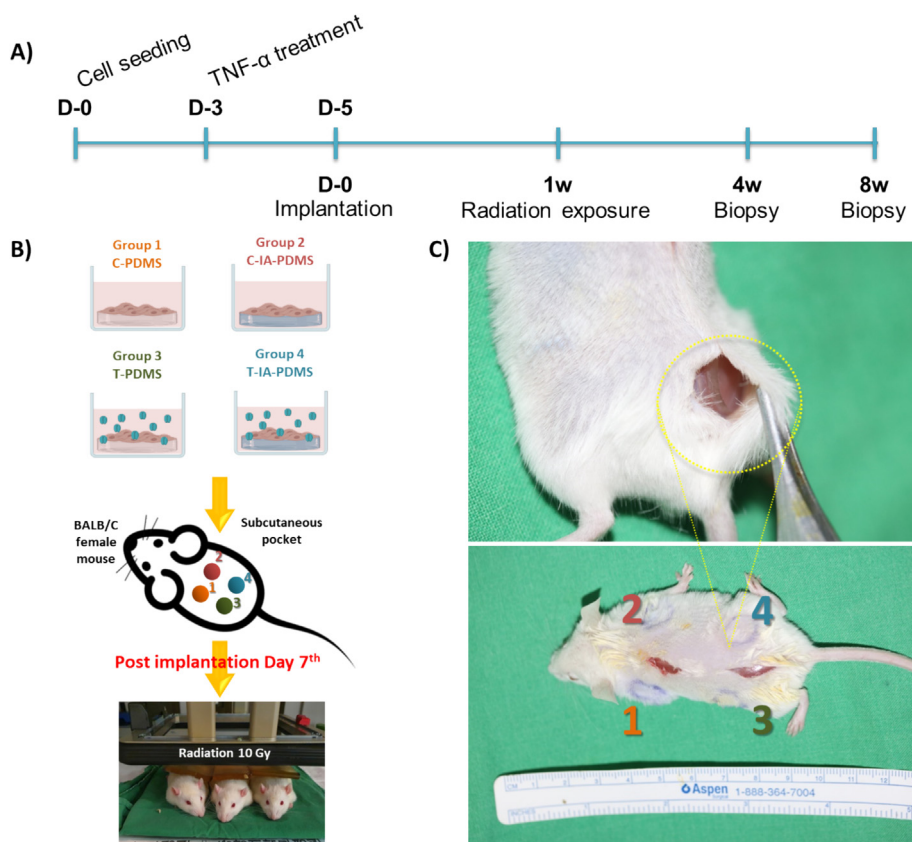


Fig. 1. Schematic of *in vivo* experiment timeline and implant composition. (A) Timeline of *in vivo* experiment, including cell culture, TNF- α treatment, implantation, and radiation exposure. (B) Composition of implants, which comprised hASCs coated on either PDMS or IA-PDMS, with or without human TNF- α (25 ng/mL) treatment. The schematic illustrates animals undergoing radiation exposure. TNF- α treatment was administered after culturing the cells on silicone membranes for 3 days, followed by implantation 48 h post-TNF- α treatment, and radiation exposure was conducted 1 week after implantation. (C) Illustration of silicone membrane implantation and the position of the implants.

expression levels to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **Table S1** demonstrates primer sequences.

Western blot analysis

To analyze specific proteins in tissue samples utilizing western blot analysis, the tissue samples were homogenized in cold RIPA buffer. The homogenates were centrifuged at 15,000 rpm at 4 °C for 30 min, and the supernatant was utilized for protein quantification with the Micro BCA™ Protein Assay Kit (Thermo Fisher) following the manufacturer's instructions. Ten micrograms of each lysate were loaded onto Any kD™ Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad, Hercules, CA) in 1x Tris/Glycine/SDS running buffer (GenDEPOT, Katy, TX) and electrophoresed at 100 V for 90 min. Subsequently, proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad) and incubated overnight at 4 °C with primary antibodies against GAPDH (1:1000, ab8245, Abcam), TGF-β1 (1:2000, ab215715, Abcam), α-SMA (1:2000, ab5694, Abcam), COL1A1 (1:2000, ab270993, Abcam), and SMAD2/3 (1:2000, ab305325, Abcam). Following primary antibody incubation, membranes were incubated with secondary antibodies (1:20000) at 25 °C for 1 h and treated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher) according to the manufacturer's instructions. Signals were detected with the ImageQuant™ LAS 4000 biomolecular imager, and protein expression was normalized to GAPDH with ImageJ software.

Histological analysis

We focused on evaluating capsule thickness to assess the effectiveness of the anti-RICC functional silicone implants. The determination of capsule thickness and inflammatory cells was performed utilizing an H&E Stain Kit (H-3502; Vector Laboratories, Inc., Newark, CA). Microscopic evaluations of the center of the capsule tissue were conducted in five fields, chosen based on minimal, intermediate, and maximal thickness, utilizing a light microscope at 40x magnification.

For the evaluation of fibroblasts, myofibroblasts, and macrophage phenotypes, immunohistochemical (IHC) staining was conducted. The primary antibodies utilized included vimentin rabbit antibody (1:250; ab92547, Abcam, Cambridge, MA), alpha-smooth muscle actin (α-SMA) mouse antibody (1:50; ab240654, Abcam), rat anti-mouse F4/80 antibody (1:66; BM8, 14–4801-82, eBioscience, San Diego, CA), rabbit anti-mouse iNOS antibody (1:100; ab15323, Abcam), goat anti-mouse Arg1 antibody (1:100; sc-18354, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-mouse IL-1R1 (M-20) antibody (1:100; sc-689, Santa Cruz Biotechnology), and goat anti-mouse MMR antibody (1:100; AF2535, R&D Systems, Inc.).

To conduct IHC labeling, the sections were placed in citrate buffer (prepared with a 10 % stock solution in distilled water, pH 6.0) and subsequently blocked with 1 % horse serum in Tris-buffered saline (pH 6.0) for 3 min. Primary antibodies were applied overnight at 4 °C, and the antibody binding was detected via polymeric techniques (UltraVision LP Detection System, TL-125-HD; Thermo Fisher Scientific). Secondary antibodies linked to horseradish peroxidase were utilized for detection, and visualization was achieved with diaminobenzidine. Photographs of the immunohistochemically stained slides were taken with a light microscope with a magnification of 200x.

TUNEL assay

To investigate apoptosis in the local tissues surrounding the implants, mice were sacrificed 24 h after radiation exposure (10 Gy). The tissues surrounding the implants were harvested,

fixed in formalin, and then embedded in paraffin to create tissue slides. Subsequently, the slides were subjected to the TUNEL assay utilizing the TUNEL assay kit (Cell Signaling Technology, Danvers, MA) following the manufacturer's instructions. The fluorescence of apoptotic cells was detected with a Leica fluorescence microscope (DM750).

Ethics statement

All human participants gave their informed consent for the scientific utilization of their samples, and all procedures involving human samples were approved by the Institutional Review Board of Chung-Ang University Hospital and adhered to the principles outlined in the Declaration of Helsinki guidelines (No. 2151–005–463).

All animal experiments and methodologies employed in this research were approved by the Institutional Animal Care and Use Committees of Chung-Ang University Hospital Institutional Review Board (Approval No. 2021–00030 and 2024–01030046).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 10 (GraphPad Software, San Diego, CA). Each analysis was based on at least three technical replicates, and the figure legends provide details on the statistical test methods employed, the biological sample size (n), and the standard deviation (SD) or standard error of the mean (SEM) values. An alpha value of at least 0.05 was utilized for all statistical analyses. All tissue images were semi-quantified in a blinded manner with ImageJ software. Measurements were based on a minimum of 20 images per group, with five randomly selected regions from each of the four animals per group.

Results and discussion

Itaconic acid conjugation improved surface hydrophilicity of silicone membranes

PDMS membranes were employed to represent silicone implants, and IA-PDMS was utilized as a hydrophilic surface-modified silicone implant. PDMS was formulated by blending the base and curing agent with a weight ratio of 10:1, which has been established as ideal for biological applications [45]. The surface of the 150 mM IA-PDMS membrane was chemically modified and utilized here. This modification has been reported to provide hydrophilic stability for up to two months, enhance biocompatibility, and reduce capsular contracture around the PDMS implant *in vivo* [28–30]. The conjugation of IA on the PDMS membrane was confirmed via water contact angle measurement and ATR/FTIR spectra analysis. The contact angle of IA-PDMS ($91.36^\circ \pm 2.72^\circ$) was significantly less than that of the PDMS membrane ($58.88^\circ \pm 2.84^\circ$; $p < 0.0001$; **Fig. S4A**). The presence of peaks at 1692 cm^{-1} and 1565 cm^{-1} in the ATR/FTIR spectra indicated the successful conjugation of IA onto the PDMS membrane surface (**Fig. S4B**). These peaks corresponded to the stretching of the C=O bonds and the formation of an amide, confirming the presence of IA on the surface of the PDMS membrane [28–30].

Confirmation of isolated hASCs

Fig. S5 presents an analysis of the phenotype of isolated hASCs at Passages 4 and 6. Utilizing fluorescence-activated cell sorting, the expression levels of surface markers, including CD45, CD73, CD90, and CD105, were evaluated. Furthermore, microscopy images provide a visual representation of the biological shape of

these cells, offering insights into their morphology at different passages. The results revealed high levels of CD73, CD90, and CD105 expression and low levels of CD45 expression (Fig. S5). This expression profile aligns with previous studies [46,47] and confirms to the normal phenotype of MSCs according to the criteria established by the International Society for Cellular Therapy [48]. The biological shapes of cells at both passages were not different, confirming their identity as hASCs utilized here.

TNF- α treatment enhances proliferation of hASCs

The impact of TNF- α on cell viability and proliferation was assessed with CCK-8 and LIVE/DEAD assays. After seeding the cells for 24 h, various doses (0, 25, 50, and 100 ng/mL) of TNF- α in complete media were applied, and cell viability was measured on Days 1, 2, 3, 5, and 7 after treatment. Based on the results, a concentration of 25 ng/mL was selected for further experiments, as it exhibited no cell cytotoxicity (cell viability > 80 % of the control) (Fig. S6).

The number of hASCs on a culture plate, PDMS, and IA-PDMS with either complete media or TNF- α (25 ng/mL)-containing complete media for 24, 48, and 72 h was determined via the linear regression equation derived from the standard curve (Fig. S1), aligning the cell quantity with the corresponding OD values

obtained from the CCK-8 assay. Notably, the number of C-hASCs on the IA-PDMS was significantly lower than those on the culture plate at 24 h ($p < 0.05$). However, at 48 and 72 h, the number of both C-hASCs and T-hASCs on the IA-PDMS membrane was comparable to those on the culture plate, while those on the PDMS exhibited significantly lower cell numbers than those on the IA-PDMS and culture plate ($p < 0.0001$; Fig. 2A). The higher cell numbers on IA-PDMS than PDMS were attributed to its surface hydrophilicity, which reduced protein adsorption and enhanced cell attachment, in line with previous findings [49]. Following TNF- α treatment for 48 and 72 h, T-hASCs exhibited significantly higher cell numbers on the culture plate and IA-PDMS surfaces than C-hASCs ($p < 0.05$). Although there was no significant difference in cell numbers on PDMS between T-hASCs and C-hASCs, a higher number of T-hASCs than C-hASCs was observed. This aligns with previous studies highlighting TNF- α 's role in promoting cell proliferation across various cell types, including hASCs [50,51]. Zubkova et al. have revealed that reactive oxygen species generation and PI3K pathway activation may contribute to the increased proliferation of TNF- α -treated ADSCs [52].

The LIVE/DEAD assay conducted on culture plates and silicone membranes confirmed the superior viability of cells on IA-PDMS compared to the PDMS membrane, maintaining comparable viability to the control (culture plate) at all evaluated time points

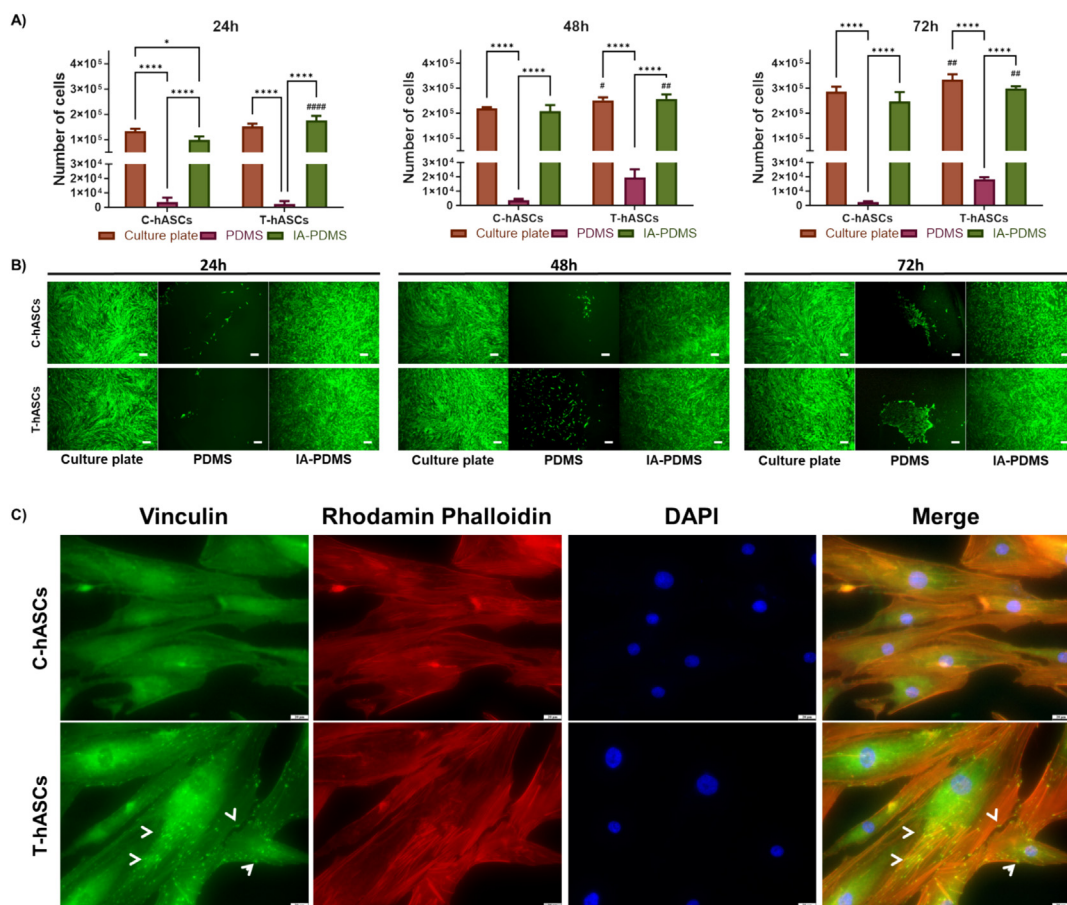


Fig. 2. Evaluation of hASCs number, viability, and actin reorganization under different conditions. (A) Number of hASCs, as determined with CCK-8 assay, cultured on culture plates, PDMS, and IA-PDMS silicone membranes at 24, 48, and 72 h. (B) Live/Dead staining images depicting cell viability of hASCs cultured on culture plates, PDMS, and IA-PDMS membranes (scale bar, 20 μ m). (C) Influence of TNF- α on actin reorganization in hASCs cultured on a chamber slide following 48 h of treatment. Cells were stained with anti-vinculin monoclonal antibody (green), indicating focal adhesion sites, Rhodamine Phalloidin (red), indicating actin filaments; and DAPI (blue), indicating nuclei. Arrows point to representative vinculin-positive cells (scale bar, 20 μ m). Cells were incubated with or without 1 mL of 25 ng/mL human TNF- α in a complete culture medium. The results are expressed as the mean $\pm$ SD ($n = 3$). *, $p < 0.05$; ****, $p < 0.001$ (two-way ANOVA, Sidak). #, $p < 0.05$; ##, $p < 0.01$; ####, $p < 0.0001$ (two-way ANOVA, Sidak; comparisons between C-hASCs and T-hASCs on the same culture surface). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2B). Moreover, on the PDMS membrane, T-hASCs exhibited enhanced attachment to the surface compared to C-hASCs, further supporting the observed cell number results.

TNF- α induces cytoskeleton rearrangement of hASCs in vitro

The reorganization of the actin cytoskeleton plays a crucial role in governing the migratory and adhesive capacities of ADSCs. Actin filaments are the architectural scaffold facilitating cellular morphological changes and efficient movement, aiding migration towards injured or compromised tissues for repair and regeneration. This reorganization allows ADSCs to leave their microenvironments and traverse through tissues. Furthermore, ADSCs require adherence to the ECM and adjacent cells to maintain tissue cohesion and optimal functionality. Actin filaments interact with diverse adhesion molecules to orchestrate cellular adhesion processes, bolstering tissue integrity and functionality [52]. Therefore, we examined the impact of TNF- α on the reorganization of the cellular actin cytoskeleton utilizing anti-vinculin antibody and rhodamine-phalloidin staining to assess the rearrangement of actin cytoskeleton in hASCs.

Fig. 2C illustrates a higher degree of actin reorganization in T-hASCs than in C-hASCs. Specifically, T-hASCs displayed discernible stress fibers and focal adhesion sites indicative of rearranged actin,

as evidenced by vinculin (green) staining, aligning with previous studies [52]. Studies involving TNF- α -treated fibroblasts [53] and endothelial cells [54] have reported similar results.

TNF- α treatment enhances inherent radiotolerance of hASCs

To evaluate the effect of radiation on hASC viability, cells were exposed to 0, 10, and 20 Gy of radiation after 48 h of TNF- α treatment. Cell viability was evaluated with the CCK-8 assay 5 days after irradiation and normalized to those of cells that were not exposed to TNF- α and radiation (control group). Fig. 3A presents the cell viability on culture plates and silicone membranes with and without radiation exposure (10 and 20 Gy). Compared to cells without radiation exposure, the cell viability of C-hASCs on the culture plate and IA-PDMS did not significantly differ after exposure to radiation at 10 Gy, indicating the radioresistance of hASCs, aligning with previous studies [55,56]. The exposure dose at 10 and 20 Gy significantly affected the viability of the C-hASCs on PDMS ($p < 0.05$), decreasing cell viability. Interestingly, the viability of T-hASCs on all culture surfaces exhibited no significant difference after exposure to radiation up to 20 Gy ($p < 0.05$), suggesting that TNF- α treatment could enhance the radiation tolerance of hASCs. The LIVE/DEAD assay images supported these findings, demonstrating greater viability of T-hASCs after radiation (Fig. 3B).

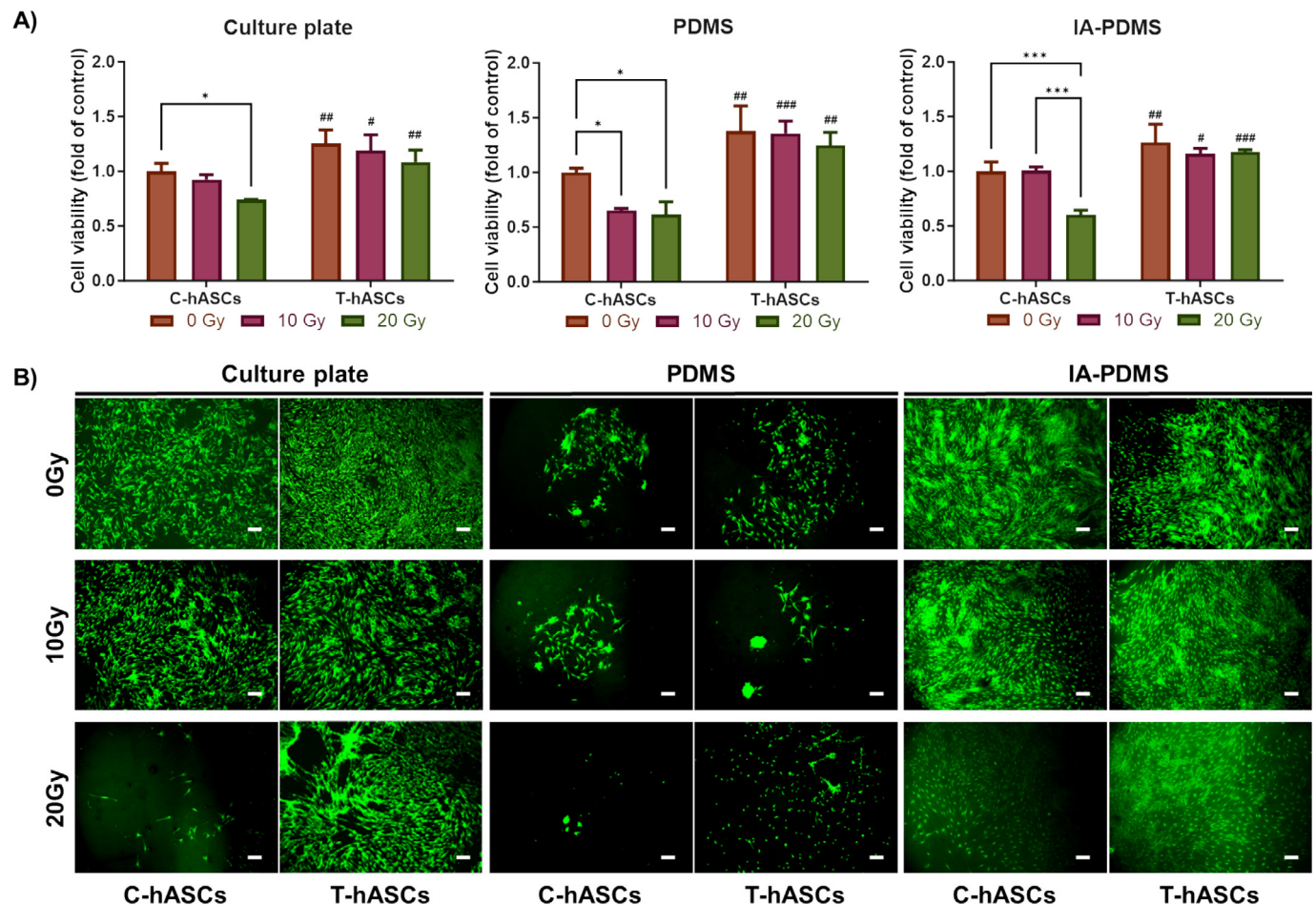


Fig. 3. Evaluation of hASC viability post-TNF- α treatment and radiation exposure. (A) Cell viability of hASCs relative to control cells (without TNF- α and radiation treatment) on each culture surface, as determined with CCK-8 assay, on Day 5 after exposure to 0, 10, and 20 Gy of radiation. (B) Live/Dead staining images depicting cell viability of hASCs cultured on culture plates, PDMS, and IA-PDMS membranes (scale bar, 20 μ m). Cells were incubated with or without 1 mL of 25 ng/mL human TNF- α in a complete culture medium for 48 h before radiation exposure. The results are expressed as the mean $\pm$ SD ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ (two-way ANOVA, Sidak). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ (two-way ANOVA, Sidak; comparisons between C-hASCs and T-hASCs at the same radiation exposure dose).

In vivo experiments

The mice were categorized into C-IA-PDMS, T-PDMS, and T-IA-PDMS as treatment groups and C-PDMS as the control group. The T-PDMS and T-IA-PDMS groups were collectively referred to as the T-groups; C-PDMS and C-IA-PDMS groups as the C-groups; C-IA-PDMS and T-IA-PDMS groups as the modified surface groups; and C-PDMS and T-PDMS groups as the non-modified surface groups. Fig. S7 illustrates the cell number and viability on the silicone membranes utilized *in vivo*, indicating a significantly higher cell number on IA-PDMS compared to PDMS ($p < 0.05$), as well as a higher cell number of T-hASCs compared to C-hASCs ($p < 0.05$).

A previous investigation convincingly demonstrated that hASC-PDMS and hASC-IA-PDMS membranes significantly reduce capsule formation around implants compared with PDMS or IA-PDMS alone [30]. To minimize the utilization of experimental animals due to ethical considerations, C-PDMS was utilized as the control instead of PDMS. This choice was made based on our hypothesis that T-groups would exhibit a greater potential in reducing capsular contracture around the implants. Each mouse received a 10-Gy radiation dose 1 week after implantation, and biopsy tissues were collected for evaluation at 4 and 8 weeks.

Capsule formation around silicone implants typically occurs within 3 weeks of implantation [57]. Hence, we designated the four-week period following implantation as the initial time point for investigating the development of capsules surrounding the silicone implants. The eight-week post-implantation period was also selected to further assess implant biocompatibility. The following section presents the results of these *in vivo* experiments.

TNF- α -treated hASCs mitigate capsule thickness

To verify the inhibitory effect of T-hASC-coated silicone implants on RICC, the thickness of the capsule was evaluated via histochemical analysis *in vivo*. Four H&E-stained images were obtained from each group to estimate the thickness of the capsules (Fig. 4A), and at least five parts of each image were analyzed. After implantation, the capsule thickness at 8 weeks increased compared to 4 weeks in all groups, with the control group demonstrating a significant increase ($p < 0.0001$) and the treatment groups not displaying significant differences (Fig. 4B). The analysis revealed a significant decrease in the thickness of the capsule in the T-groups compared with the C-groups at 4 and 8 weeks after implantation ($p < 0.0001$; Fig. 4B). Notably, the modified surface groups exhibited a more pronounced reduction in capsule thickness compared with the non-modified surface groups ($p < 0.0001$), with the T-groups exhibiting a significantly greater reduction ($p < 0.0001$).

TNF- α -treated hASCs alleviate inflammatory response

An initial phase of the cellular response during the fibrosis progression involves the inflammatory reaction, which encompasses multiple responses featuring diverse inflammatory cell types, including lymphocytes, neutrophils, eosinophils, and basophils. These inflammatory cells influence macrophage activities, leading to their fusion into foreign body giant cells and eventual fibrosis formation [58]. Fig. 4A and C, as well as Fig. S8, present representative images and semi-quantitative analysis of the inflammatory cells in the tissue samples surrounding the implant at 4 and

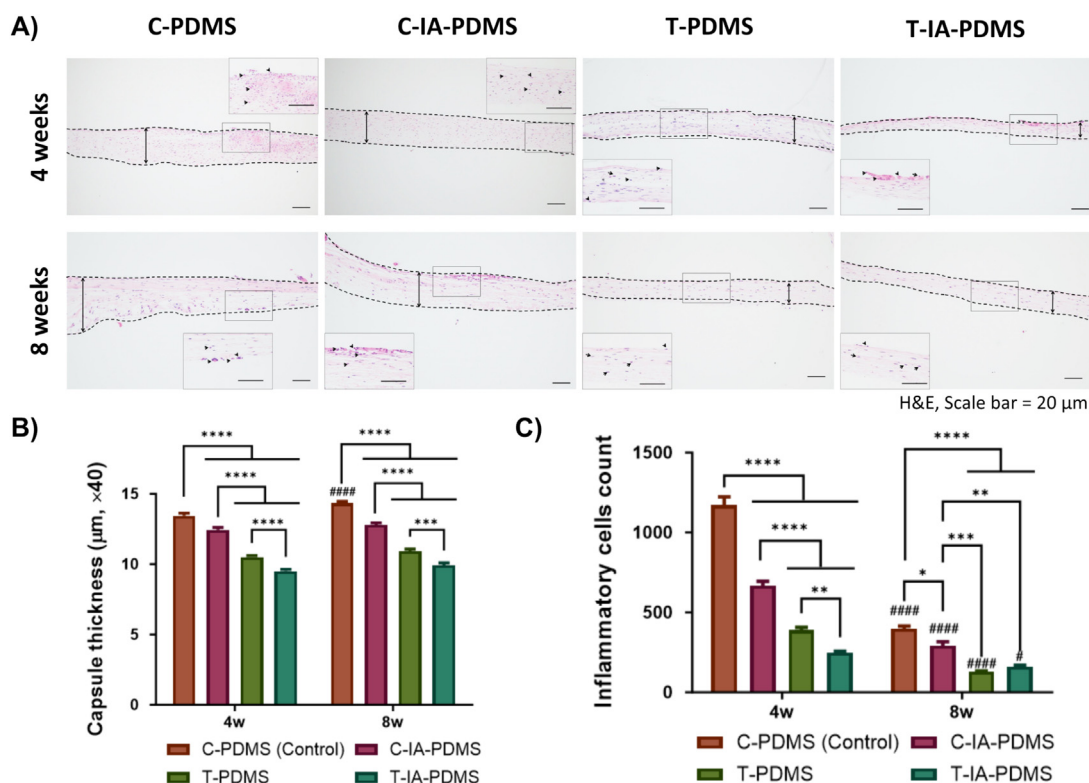


Fig. 4. Evaluation of *in vivo* formation of fibrous capsules and inflammatory cells surrounding different silicone membrane implants. (A) Representative images depicting capsular formation and inflammatory cells surrounding hASC-coated PDMS (C-PDMS), hASC-coated IA-PDMS (C-IA-PDMS), TNF- α -treated hASC-PDMS (T-PDMS), and TNF- α -treated hASC-IA-PDMS (T-IA-PDMS) implants at 4 and 8 weeks after implantation (scale bars, 20 μ m). Inset images depict a magnified view. Double-headed arrows indicate the capsule thickness, and short arrows indicate representative inflammatory cells. (B) Semi-quantitative analysis of capsule thickness. (C) Semi-quantitative analysis of inflammatory cells. The results are expressed as the mean $\pm$ SEM ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (two-way ANOVA, Bonferroni). #, $p < 0.05$; ####, $p < 0.0001$ (two-way ANOVA, Bonferroni; comparisons of tissue surrounding the same implant group between 4 weeks and 8 weeks post-implantation).

8 weeks post-implantation employing H&E staining. Pronounced inflammation was observed in the control group, whereas a significant reduction in inflammation was evident in the treatment groups compared to the control group from the outset ($p < 0.0001$; Fig. 4C). The degree of inflammation in all groups significantly decreased at 8 weeks compared to 4 weeks ($p < 0.05$). This result is consistent with our previous study, which demonstrated a decrease in inflammation of hASC-coated implants at 8 weeks compared to 2 and 4 weeks [30]. Notably, a lower degree of inflammation was observed in the T-groups compared to the C-groups at both time points ($p < 0.001$). Hence, we hypothesized that the environment in the T-groups was more biocompatible.

TNF- α -treated hASCs minimize fibroblasts and myofibroblasts

Myofibroblasts, essential for fibrogenesis and derived from fibroblasts, persist in radiation injury, contributing to an abnormal ECM buildup [59,60]. The transition of fibroblasts to myofibroblasts leads to increased tension and contributes to the development of capsular contracture [61]. The fibrosis assessment often involves determining the number of myofibroblasts [62]. An elevated presence of myofibroblasts within the capsule induces heightened contraction, applying increased pressure on the implant and causing tissue deformation and pain. The differentiation of fibroblasts into myofibroblasts is mediated by fibroblast-derived TGF- β 1 via α -SMA synthesis, which subsequently plays a significant role in promoting capsular contracture [63]. IHC images of vimentin-stained cells (positive for fibroblasts) and α -SMA (positive for myofibroblasts) were analyzed to assess fibrosis formation around implants in mouse tissues. As illustrated in Fig. 5, fibroblasts and myofibroblasts were predominantly adjacent to the

implant. The semi-quantitative analysis revealed significantly fewer fibroblasts ($p < 0.0001$; Fig. 5A and B) and thinner myofibroblast layers ($p < 0.0001$; Fig. 5C and D) in the T-groups than in the C-groups at 4 and 8 weeks.

To verify the tissue-staining results, we determined the expression of pro-fibrosis genes, including collagen 1 alpha 1 (COL1A1), collagen 3 alpha 1 (COL3A1), α -smooth muscle actin (α -SMA), suppressor of mothers against decapentaplegic 3 (SMAD3), and transforming growth factor β 1 (TGF- β 1), in capsule tissues at 4 and 8 weeks after implantation via qPCR analysis. At 4 weeks post-implantation, the analysis of pro-fibrosis gene expression levels between groups revealed significantly lower expression levels in the treatment groups compared to the control group ($p < 0.05$; Fig. 6A–E).

After implantation, the expression of almost all pro-fibrosis genes decreased at 8 weeks compared to 4 weeks, except for COL3A1, where gene expression increased at 8 weeks compared to 4 weeks. The expression of COL1A1 and COL3A1 plays a pivotal role in fibrogenesis, characterized by the pathological accrual of collagen and additional ECM constituents within tissues. At 4 and 8 weeks after implantation, the results demonstrated significant suppression of COL1A1 and COL3A1 expression in the treatment groups ($p < 0.05$; Fig. 6A and B), particularly in the T-IA-PDMS group, indicating reduced collagen formation around the implant and, thus, suppressed fibrosis. However, significant increases in COL3A1 expression were observed in the C-groups at 8 weeks after implantation ($p < 0.05$; Fig. 6B). This suggests prolonged tissue remodeling [64,65], possibly due to unresolved inflammation or foreign body reactions. In contrast, the T-groups exhibited no significant difference in COL3A1 expression levels, indicating effective modulation of collagen remodeling and decreased risk of capsular

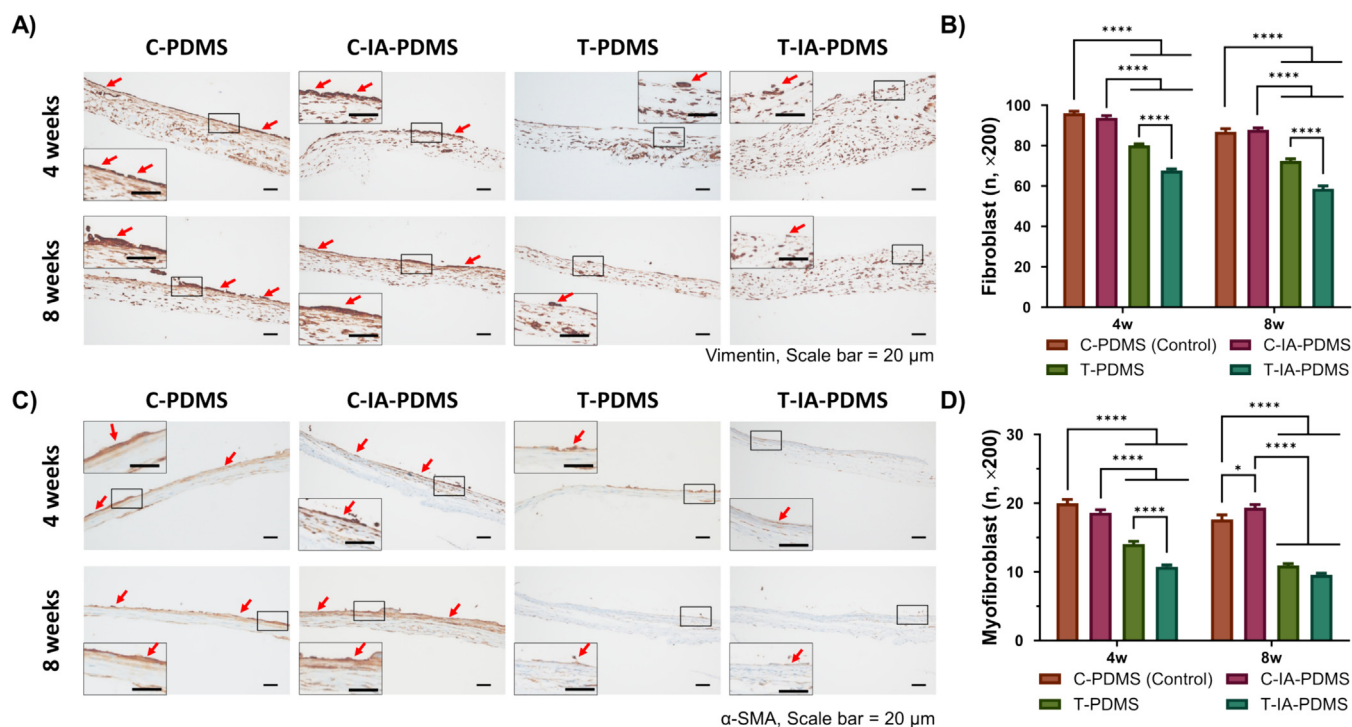


Fig. 5. In vivo evaluation of fibroblasts and myofibroblasts in tissues surrounding different silicone membrane implants. (A, C) Representative immunohistochemistry (IHC) images of (A) fibroblasts-stained tissues and (C) myofibroblasts-stained tissues surrounding hASC-coated PDMS (C-PDMS), hASC-coated IA-PDMS (C-IA-PDMS), TNF- α -treated hASC-PDMS (T-PDMS), and TNF- α -treated hASC-IA-PDMS (T-IA-PDMS) implants at 4 and 8 weeks post-implantation (scale bars, 20 μ m). Brown dense layers indicate vimentin-positive fibroblast-stained tissues, and brown stained layers indicate α -SMA-positive myofibroblasts-stained tissues. Red arrows indicate representative vimentin-positive and α -SMA-positive myofibroblasts-stained tissues. (B, D) Semi-quantification of (B) vimentin-positive fibroblasts and (D) α -SMA-positive myofibroblasts. Data are presented as the mean $\pm$ SEM ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (two-way ANOVA, Bonferroni). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

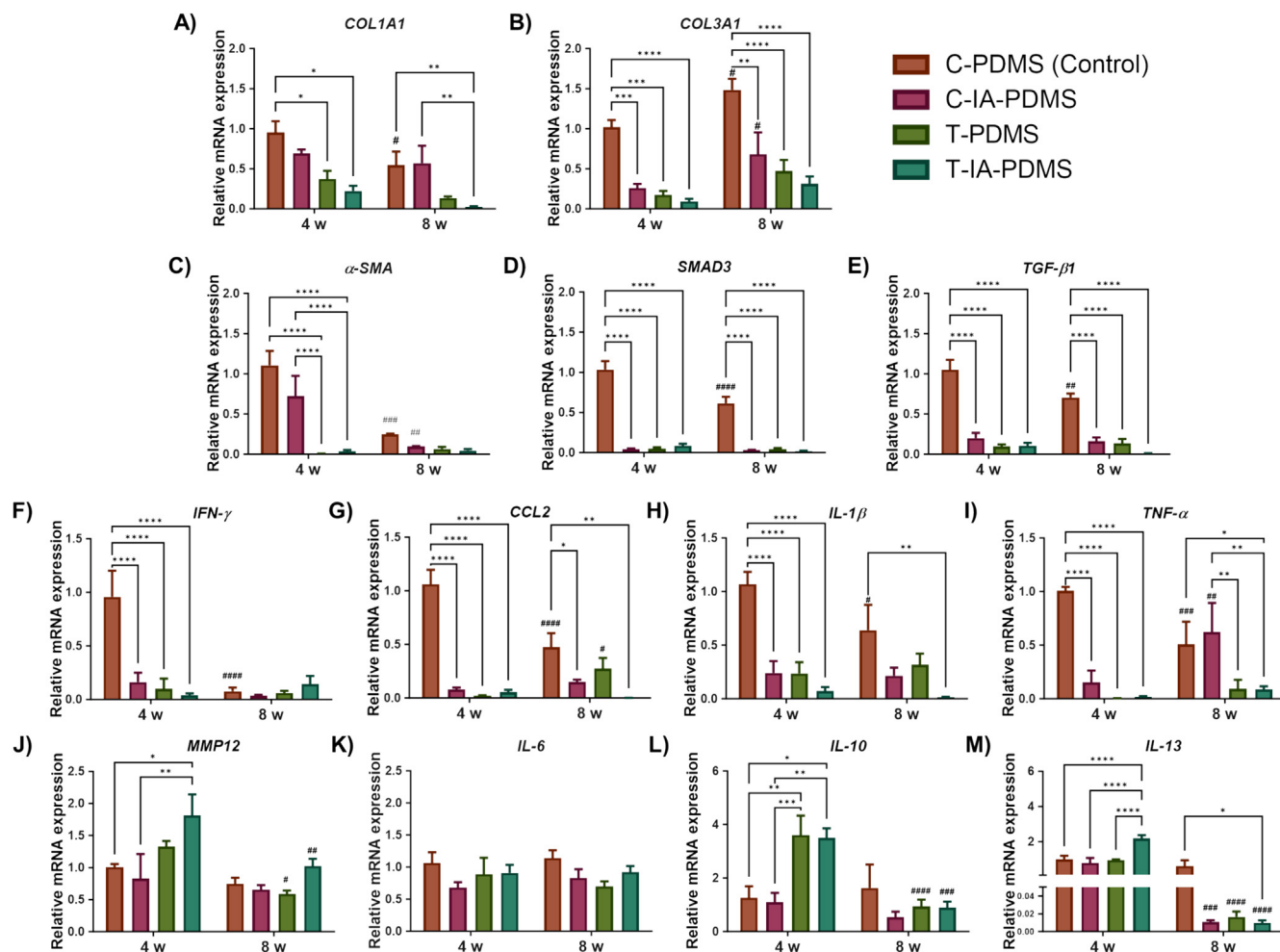


Fig. 6. mRNA expression in tissues surrounding different silicone membrane implants at 4 and 8 weeks post-implantation. Reverse transcription-quantitative real-time PCR analysis of *in vivo* expression of (A–D) fibrosis-related genes and (F–I) M1 and (J–M) M2 macrophage polarization-related genes. mRNA expression levels were normalized to GAPDH and expressed relative to the control group at each time point. The results are expressed as the mean $\pm$ SEM (n = 4). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (two-way ANOVA, Bonferroni). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$ (two-way ANOVA, Bonferroni; comparisons of tissue surrounding the same implant group between 4 weeks and 8 weeks post-implantation).

contracture. This may result in the accumulation of type III collagen and a less organized fibrous capsule around the implant in the C-groups compared to the T-groups.

The regulation of these gene expressions is orchestrated by diverse signaling pathways and cytokines, among which TGF- β 1 is central in stimulating their expression and instigating the differentiation of fibroblasts into myofibroblasts. This mechanism contributes to the regulation of ECM remodeling, and disruptions in this balance have been observed during irradiation [66], leading to excessive collagen production in fibrotic tissue [67]. Research has demonstrated that α -SMA correlates with fibroblast contractility and undergoes upregulation during the differentiation of fibroblasts into myofibroblasts [68,69]. Our study demonstrated significant suppression of α -SMA in tissues around the implant in the treatment group ($p < 0.0001$; Fig. 6C), consistent with the IHC-staining result where a reduction of α -SMA was notably observed in the T-groups (Fig. 5C and D). In addition, research findings suggest that activating the TGF- β 1 signaling pathway promotes the synthesis of α -SMA via the mediation of SMAD3 [70]. Hence, we investigated the expression levels of TGF- β 1 and SMAD3, observing significantly lower levels in the treatment groups at both time points ($p < 0.0001$; Fig. 6D and E). Although semi-quantitative staining results confirmed that T-IA-PDMS exhibited the lowest fibroblast and myofibroblast count, the data on the suppression

of pro-fibrosis gene expression indicated no significant difference among the treatment groups.

In addition, we confirmed the expression of TGF- β 1, SMAD3, α -SMA, and COL1A1 at the protein level with western blot analysis. After implantation, protein expression levels in all groups decreased at 8 weeks compared to those at 4 weeks. The treatment groups exhibited significant decreases in TGF- β 1, α -SMA, and SMAD3 expression at 4 weeks post-implantation and decreases in TGF- β 1 and SMAD3 at 8 weeks post-implantation compared to the control group ($p < 0.05$; Fig. S9A–E). Although COL1A1 did not exhibit significant changes at both time points, the treatment groups exhibited a reduced trend compared to the control group. These protein expression patterns were consistent with the results obtained via RT-qPCR analysis (Fig. 6A–E).

These findings indicated that the treatment groups inhibited fibrosis formation and myofibroblast accumulation, contributing to the prevention of capsular contracture.

TNF- α -treated hASCs modulate macrophage polarization toward M2 type

Capsular contracture development is influenced by the inflammatory reaction triggered by implants and radiation, and macrophages play a pivotal role in this context. Macrophages can

exhibit different phenotypes based on their microenvironment and signals. The classic M1 macrophage phenotype is correlated with inflammation and tissue damage, while the alternative M2 phenotype is associated with tissue repair and regeneration [71]. Ensuring a harmonious balance between M1 and M2 macrophage polarization is essential in shaping the outcome of the inflammatory response and tissue integration surrounding the implant.

Previous research has demonstrated that hASCs can promote M2 macrophage polarization [72,73], which is associated with tissue repair and reduced fibrosis, suggesting their potential for inhibiting capsular contracture. Accordingly, we examined the influence of T-hASC-coated implants on macrophage polarization via IHC staining of tissues surrounding the implants at 4 weeks post-implantation. This was accomplished by comparing the expression levels of pro-inflammatory (M1) markers, including inducible nitric oxide synthase (iNOS) and IL-1 receptor 1 (IL-1R1), with anti-inflammatory (M2) markers, including macrophage mannose receptor (MMR) and arginase-1 (Arg-1). Fig. 7 presents representative IHC images and semi-quantitative data depicting macrophages expressing M1 and M2 markers in the tissue surrounding implants. As expected, macrophages expressing both M1 and M2 markers were present in all groups. However, IHC staining revealed a significant decrease in M1 macrophages (positive for iNOS and IL-1R1) and an increase in M2 macrophages (positive for MMR/CD206 and Arg1) in the T-group compared to the C-group ($p < 0.0001$; Fig. 7). These results were further confirmed via gene expression analysis of macrophage polarization.

To verify and assess the response of macrophages to implantation at the molecular level, we examined the expression levels of genes associated with macrophage polarization, including matrix metalloproteinase 12 (MMP12), interferon γ (IFN- γ), chemokine ligand 2 (CCL2), TNF- α , interleukin 1 β (IL-1 β), IL-13, IL-6, and IL-10. In all

groups, the expression of M1 and M2 genes in the tissue surrounding the implant decreased at 8 weeks compared to 4 weeks after implantation (Fig. 6J–M). There was no significant change in IL-6 expression at either time point (Fig. 6K). At 4 weeks after implantation, a significant reduction in M1 gene expression (IFN- γ , CCL2, TNF- α , and IL-1 β ; $p < 0.0001$; Fig. 6F–I) and a significant rise in M2 gene expression (MMP-12, IL-13, and IL-10; $p < 0.05$; Fig. 6J, L, and M) were observed in the tissues surrounding the implants in the treatment groups. TNF- α plays a dual role in the breast cancer tumor microenvironment [68,69]. TNF- α expression levels confirmed that TNF- α treatment did not involve upregulation of TNF- α *in vivo* (Fig. 6I). Notably, at 4 weeks after implantation, the T-IA-PDMS group exhibited a significant upregulation in M2 gene expression (MMP-12, IL-13, and IL-10; $p < 0.05$; Fig. 6J, L, and M) compared to the other groups.

TNF- α -treated hASCs suppress radiation-induced apoptosis *in vivo*

Exposure to ionizing radiation during cancer treatment can induce cellular damage, often resulting in apoptosis. This phenomenon occurs in various cell types within the targeted tumor and the surrounding healthy tissues. The resulting tissue damage can disrupt the normal healing process, potentially leading to increased fibrosis and scar tissue formation, contributing to capsular contracture development [74]. Moreover, TNF- α has been reported to induce cell apoptosis [75]. Therefore, to assess the impact of radiation on apoptosis in the tissue surrounding the implants and to confirm that the hASC treatment with TNF- α did not induce cell apoptosis owing to the treated TNF- α , a TUNEL assay was conducted. Representative fluorescence images of TUNEL-positive apoptotic cells are presented in Fig. 8A. At 24 h post-irradiation, the percentage of apoptotic cells was significantly

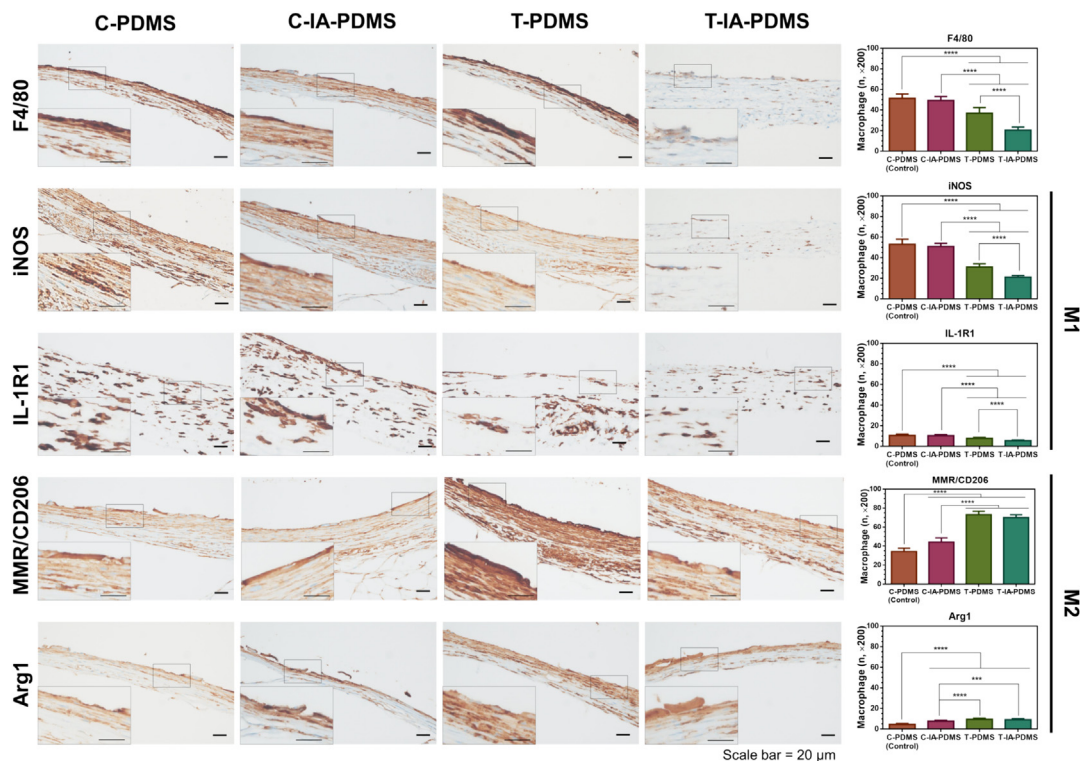


Fig. 7. Evaluation of *in vivo* macrophage polarization in tissues surrounding different silicone membrane implants at 4 weeks post-implantation. (Left) Representative IHC images of tissues around the implants reveal brown-stained layers indicating positive macrophage markers (scale bars, 20 μ m). Inset images represent a magnified view. (Right) Semi-quantification of IHC-stained macrophages is also presented. The results are expressed as the mean $\pm$ SEM (n = 4). ***, $p < 0.001$; ****, $p < 0.0001$ (one-way ANOVA, Bonferroni).

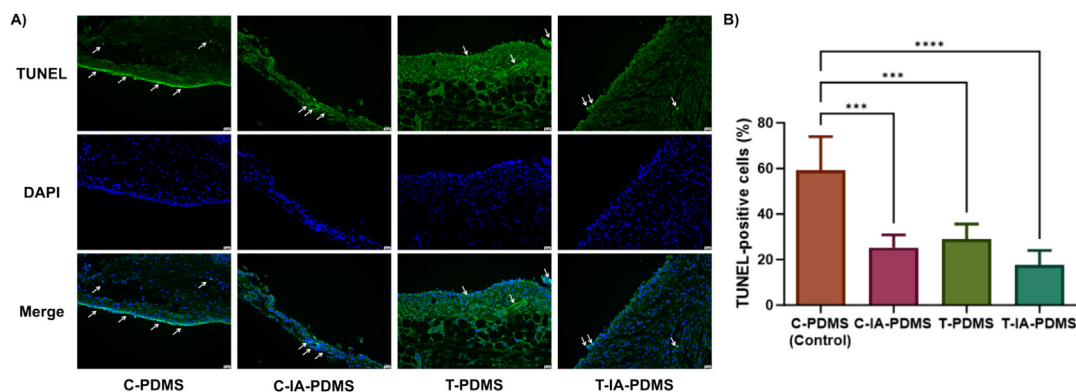


Fig. 8. Evaluation of radiation-induced cell apoptosis in tissues surrounding different silicone membrane implants after expose to radiation for 24 h. (A) Representative images and **(B)** semi-quantitative analysis of TUNEL-positive stained apoptosis cells. TUNEL-positive cells were stained green, and nuclei were stained blue (scale bar, 20 μ m). Arrows point to representative TUNEL-positive cells. The staining intensity of TUNEL-positive and DAPI-positive nuclei were counted, and data was expressed as a percentage of the total number of cells [TUNEL-positive cells (%) = fluorescent intensity of (TUNEL-positive cell/DAPI-positive cell)]. The results are expressed as the mean $\pm$ SEM ($n = 4$). ***, $p < 0.001$; ****, $p < 0.0001$ (two-way ANOVA, Bonferroni). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower in the treatment groups than in the control group ($p < 0.001$; Fig. 8B). While there was no significant difference among the treatment groups, the tissue around the T-IA-PDMS group exhibited the lowest percentage of apoptotic cells. The significantly lower percentage of apoptotic cells in the tissue around the C-IA-PDMS group than the C-PDMS group might result from a higher cell number in the C-IA-PDMS group, thereby reducing cell apoptosis. Although the count of hASCs on T-PDMS was lower than that on C-IA-PDMS (Fig. S7), the percentage of apoptotic cells in the tissue surrounding the T-PDMS group was not significantly different from those in C-IA-PDMS and T-IA-PDMS.

These results indicate that the utilization of TNF- α in the treatment of hASCs did not induce TNF- α -mediated cell apoptosis. Conversely, it suggests an enhanced potential of hASCs in reducing radiation-induced cell apoptosis. ADSCs have been reported to reduce radiation-induced apoptosis, potentially preventing radiation-induced tissue injury [76,77]. Our results suggest that TNF- α treatment of hASCs, especially on T-IA-PDMS, could effectively suppress radiation-induced apoptosis *in vivo*, potentially reducing normal tissue damage and subsequently mitigating RICC.

Assessment of growth factors released from TNF- α -treated hASCs

We further investigated the profile of growth factors released from T-hASCs *in vitro*. We utilized growth factor arrays to assess the signaling molecules involved in the paracrine effect on alleviating capsular contracture *in vivo*. A notable abundance of VEGF was detected in T-hASCs, which was significantly higher than that released from C-hASCs ($p < 0.0001$; Fig. 9A–D, and Fig. S10A–C). This suggested that TNF- α treatment enhanced VEGF secretion from hASCs. Previous reports have demonstrated that ADSCs exhibit a paracrine action involving the release of substantial quantities of VEGF [78–80]. Furthermore, it has been reported that TNF- α enhances the therapeutic potential of ADSCs by stimulating the secretion of various factors, including VEGF [81], thereby supporting our findings. The released VEGF acts on ADSCs in an autocrine manner, stimulating their proliferation. This mechanism is supported by previous studies demonstrating that VEGF enhances the proliferation of ADSCs [82,83].

Next, we confirmed the VEGF release profile in a radiation exposure environment by comparing the released VEGF from C-hASCs and T-hASCs after radiation exposure at 0 and 10 Gy. The culture media of C-hASCs and T-hASCs cultured on a culture plate and silicone membranes were collected 72 h after radiation exposure and

utilized to assess the levels of released VEGF with an ELISA kit. The concentration of human VEGF in the collected medium was quantified utilizing the linear regression equation derived from the standard curve (Fig. S3). T-hASCs exhibited significantly higher VEGF release than C-hASCs on all surfaces with and without radiation exposure ($p < 0.05$; Fig. 9E–G). Even on the PDMS membrane, which exhibited a low number of hASCs, a significantly higher amount of VEGF release from T-hASCs compared to C-hASCs was also observed ($p < 0.05$; Fig. 9F). This result indicated that radiation did not affect the VEGF production from T-hASCs.

The autocrine feedback mechanism, wherein ADSCs produce VEGF to support their own growth and survival, may contribute to the enhanced survival of T-hASCs after radiation exposure. We also found that T-hASCs exhibited significantly higher secretion levels of IL-6 and IL-8 than C-hASCs ($p < 0.05$; Fig. S10A–C). This increase in VEGF, IL-6, and IL-8 released from hASCs aligns with a previous study [52], which reported that TNF- α upregulated these factors in ADSCs at both mRNA and protein levels. These cytokines, along with VEGF, have been reported to be associated with radiation tolerance [84–86]. Therefore, the increased secretion of these cytokines and growth factors from T-hASCs might contribute to the observed radioprotective effects. However, investigating the changes in the levels of other growth factors and cytokines in the culture medium of T-hASCs compared to C-hASCs would be important for further studying the paracrine signaling of these cells in various applications.

Overall, we investigated the complex interplay between mastectomy with silicone implants and radiation therapy. While radiation therapy is crucial for targeting residual cancer cells after mastectomy, it can induce fibrosis and elevate the risk of capsular contracture around breast implants. To address this issue, we treated hASCs with TNF- α to study its paracrine effect on RICC.

In vitro, the hydrophilic surface modification of implants improved cell viability and attachment (Fig. 2). However, radiation adversely affected cell viability in the absence of TNF- α treatment (Fig. 3), indicating potential benefits when applied *in vivo*. *In vivo*, T-hASCs significantly suppressed capsular contracture around silicone implants when radiation therapy was employed, suggesting their potential for inhibiting RICC. This remarkable outcome can be attributed to the superior performance of T-hASC-coated implants in the reduction of fibrosis-related factors, including gene and protein expression (Fig. 6 and Fig. S9), myofibroblasts, and fibroblast cells (Fig. 5), as well as capsule thickness at the tissue-implant interface (Fig. 4). In addition, the implants coated with

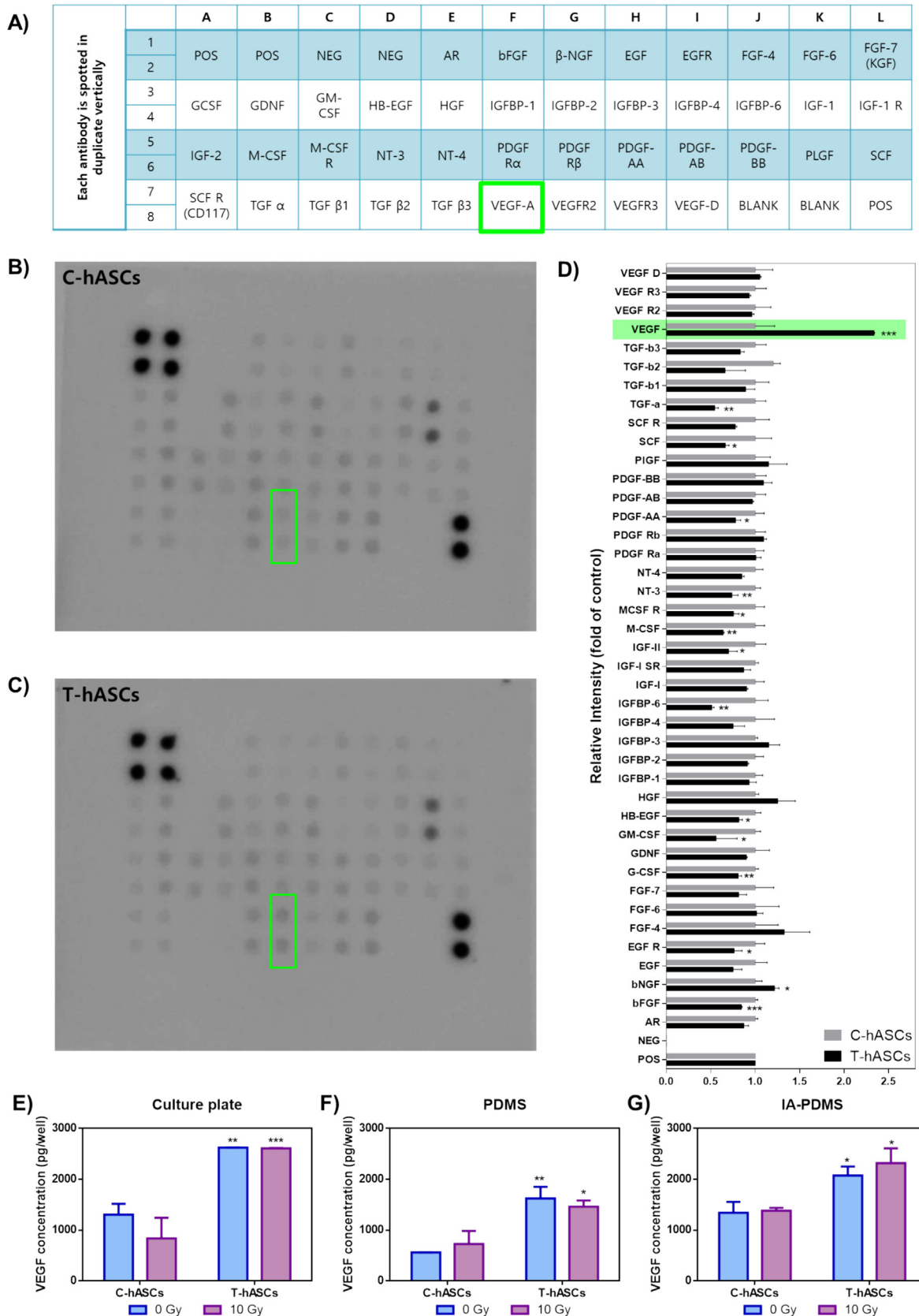


Fig. 9. Growth factor protein analysis in conditioned medium from hASCs after treatment with human TNF- α (25 ng/mL) for 72 h utilizing Human Growth Factor Array C1. (A) Antibody mapping of C1 array. Chemiluminescence images of microarrays of cultured medium (B) without TNF- α and (C) with TNF- α . (D) Quantitative relative intensity analysis of growth factors released from hASCs with and without TNF- α , determined by ImageJ binary pixel intensity. The results are expressed as the mean $\pm$ SDs (n = 6). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (multiple t -test). (E) Levels of VEGF release from cultured hASCs on culture plates and indicated types of silicone membranes incubated with and without 1 mL of 25 ng/mL human TNF- α in a complete culture medium for 48 h before radiation exposure, as assessed with an ELISA kit. The evaluations were performed on Day 5 after exposure to 0 and 10 Gy of radiation. Data are presented as means $\pm$ SDs (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ (two-way ANOVA, Sidak, comparisons between C-hASCs and T-hASCs at the same radiation exposure dose).

T-hASCs demonstrated an enhanced capacity to modulate the immune response by alleviating inflammatory cells (Fig. 4A and C) and radiation-induced apoptosis (Fig. 8), attenuating the polarization of M1 macrophages, and promoting the polarization of M2 macrophages (Fig. 6J–M and Fig. 7), resulting in the suppression of fibrosis.

We further found that treating hASCs with TNF- α increased VEGF production. Notably, TNF- α treatment provided radioprotective effects on hASCs without compromising VEGF release (Fig. 9), presenting a novel technique to enhance the cells' radioprotective and regenerative capabilities. The enhanced presence of T-hASCs on the hydrophilic modified surface (IA-PDMS) led to higher VEGF release than T-hASCs on the hydrophobic surface (PDMS). This increase in VEGF release facilitated a paracrine effect of the hASCs, contributing to a thinner capsule *in vivo* (Fig. 4A and B). These findings suggest that T-hASCs exhibit more potential in suppressing RICC than C-hASCs, representing a novel approach to enhancing cell-based therapies for radiation-damaged tissues and underscoring the importance of preconditioning cells to withstand harsh post-surgical environments.

Studies have reported the ability of ADSCs to facilitate wound healing in irradiated tissue [87,88], a process in which VEGF plays a crucial role by promoting angiogenesis and supporting blood vessel development and sustenance [89]. In our study, we speculate that the increase in VEGF likely facilitated angiogenesis and tissue repair, supporting the regenerative properties of hASCs and surrounding tissues after radiation exposure. This speculation is supported by previous findings demonstrating that TNF- α -treated ADSCs enhance the growing capacity of microvessels [52]. In addition, Heo et al. reported that treating ADSCs with TNF- α accelerates cutaneous wound healing via paracrine mechanisms involving IL-6 and IL-8 [39]. Similarly, in our study, T-hASCs exhibited increased release of IL-6 and IL-8 in the culture medium, as detected by the cytokine array (Fig. S10).

Our findings suggest that TNF- α treatment enhances the radiation tolerance of hASCs, thereby preventing RICC while simultaneously reducing inflammation and facilitating tissue healing around the implant. We speculate that the released VEGF enhances tissue integration, stimulating angiogenesis and supporting the regenerative properties of hASCs and surrounding tissues after radiation exposure. The observed higher stress in the arrangement of the actin cytoskeleton of T-hASCs compared to C-hASCs supports this speculation (Fig. 2C), as the rearrangement of the actin cytoskeleton facilitates cell migration to injured tissues and helps maintain tissue integrity and function.

A more pronounced reduction in RICC was observed in the T-groups, especially the T-IA-PDMS group. The significantly higher release of VEGF from T-hASCs than C-hASCs ($p < 0.001$, Fig. 9) might contribute to tissue regeneration and M2 polarization of T-groups *in vivo*, as VEGF has been reported to promote M2 macrophage polarization [90]. In addition, ADSCs have been reported to influence M2 polarization [91]. Our previous study demonstrated that thinner capsule formation is associated with hASC-coated implants, possibly due to increased M2 macrophage polarization [30]. Here, radiation-tolerant T-hASCs influenced the differentiation of M1 macrophages into M2 macrophages, which are involved in both foreign body reactions and radiation-induced inflammatory reactions. This led to an earlier onset of the healing process, reduced infiltration of immune-related cells, and subsequently prevented RICC.

As illustrated in Fig. 6M, the T-IA-PDMS group exhibited significantly higher IL-13 expression at 4 weeks post-implantation than other groups ($p < 0.0001$). This resulted in higher levels of MMP12 and IL-10 ($p < 0.05$; Fig. 6J and L), which are products of M2 macrophages [92,93]. IL-13 facilitates the differentiation of macrophages towards an M2 phenotype [94]. The assessment of cytokine release

from hASCs and T-hASCs *in vitro* aligned with the mRNA expression results, exhibiting higher release of IL-13 and IL-10 from T-hASCs compared to C-hASCs, as presented in Fig. S10.

Furthermore, IL-6 has been reported to participate in the polarization process of M1 into M2 macrophages [95]. Although there was no significant difference in IL-6 expression between C-groups and T-groups *in vivo* (Fig. 6K), the *in vitro* cytokine release results revealed that T-hASCs significantly released higher IL-6 than C-hASCs ($p < 0.05$, Fig. S10). We assume that evaluating macrophage polarization at the gene expression level 4 weeks after implantation affected the assessment of the macrophage response, as capsule formation around silicone implants typically occurs within 3 weeks after implantation [57], thus exhibiting no significant difference in IL-6 expression.

These cytokine and growth factor release profiles supported the mechanism of M2 polarization of T-hASCs here. This observation highlights the potential of T-hASCs to modulate the immune response and promote tissue repair more effectively than untreated hASCs. However, abundant VEGF production and the presence of other released cytokines before radiation therapy can have both beneficial and detrimental effects [96]. Further research is necessary to comprehensively understand the impact of VEGF and other released cytokines, which are paracrine products from T-hASCs, on the radiation tolerance capacity of hASCs and treatment outcomes in radiation-related conditions.

Although our study did not conduct *in vivo* tracking of hASCs, the significant differences in RICC prevention between C-hASCs and T-hASCs, as well as between cells cultured on PDMS and IA-PDMS surfaces, support the idea that either the autocrine effect of the cells themselves or their paracrine effect on implants influenced a reduction of RICC. Previous research has demonstrated variations in capsular contracture inhibition between implants with and without hASCs coating after 8 weeks of implantation [30]. Furthermore, Elshaer et al. reported therapeutic benefits of intravitreal injection of either ADSCs or conditioned medium of ADSCs in alleviating retinal complications of diabetes in a mouse model three weeks post-injection compared to the control group [97].

While these studies support our assumption that hASCs and/or their paracrine effects offer *in vivo* therapeutic benefits and biocompatibility, additional investigations are warranted. Conducting *in vivo* tracking of hASCs, coupled with a comprehensive exploration of the radiation tolerance capacity of T-hASCs—including mechanisms of action, examination of radiation-sensitive biomarkers, and assessment of chromosome aberrations—would provide valuable evidence to further validate the influence of T-hASCs on their therapeutic potential. In addition, quantifying VEGF levels *in vivo* could offer further insights into the mechanisms underlying the therapeutic effects of T-hASCs.

Despite our murine model providing informative results, it may not fully capture the complexities inherent in human breast reconstruction. Therefore, subsequent investigations, including larger animal models and human clinical trials, are warranted to enhance the generalizability of our findings. Our study, which focuses on a specific application of T-hASCs-coated implants, underscores the necessity for probing the long-term effects and sustainability of the resulting anti-fibrotic and anti-inflammatory environment. Future research in the cancer model, involving mastectomy, reconstruction, and radiation therapy, with a focus on the tissue regeneration capabilities of T-hASCs after radiation therapy, could provide valuable insights, advancing the biomedical and clinical applications of T-hASCs.

Based on the outcomes of our investigation, the utilization of autologous cells extracted from the patient's adipose tissue is anticipated to represent an ideal treatment method for regenerating tissue damage in actual clinical practice. Current therapeutic

options for this complication are limited, and our study introduces a novel and promising approach to overcome this challenge, representing a groundbreaking advancement in the treatment of RICC. By leveraging the radioprotective properties of TNF- α -treated hASCs and combining them with hydrophilic surface-modified silicone implants, we offer a pioneering strategy to mitigate fibrosis and enhance tissue regeneration after mastectomy and radiation therapy. This personalized approach harnesses the inherent advantages of autologous transplantation, ensuring compatibility and minimizing the risk of adverse reactions. It also holds the potential to significantly improve patient outcomes in terms of both efficacy and cost-effectiveness.

Conclusion

Our comprehensive investigation reveals the potential of T-hASCs as a promising intervention for mitigating RICC surrounding silicone implants post-mastectomy. The hydrophilic surface modification of implants enhanced cell viability and attachment *in vitro*, but radiation without TNF- α treatment adversely affected cell viability. *In vivo*, T-hASC-coated implants significantly reduced capsular contracture because of their superior performance in reducing fibrosis-related factors, modulating the immune response by promoting M2 macrophage polarization and alleviating radiation-induced apoptosis. TNF- α treatment increased VEGF production without compromising the radioprotective effects, further enhancing the therapeutic potential of T-hASCs over C-hASCs. This multifaceted approach promises to enhance post-reconstruction outcomes and offers a cost-effective solution that minimizes the risk of adverse reactions.

Despite these promising results, additional investigations are necessary to validate the therapeutic effects of T-hASCs in future research, including *in vivo* tracking of hASCs, exploration of radiation tolerance mechanisms, and quantification of VEGF levels. Furthermore, focusing on the long-term effects and sustainability of the anti-fibrotic and anti-inflammatory environment created by T-hASCs, particularly in the context of mastectomy, reconstruction, and radiation therapy, along with larger animal models and human clinical trials, is essential to confirm our findings and enhance their generalizability.

While the therapeutic option of RICC is limited, our findings suggest that utilizing autologous cells from a patient's adipose tissue could represent an ideal approach for regenerating tissue damage in clinical practice, leveraging the advantages of personalized treatment. This study provides valuable insights into regenerative medicine and breast cancer treatment complications, ultimately enhancing breast reconstruction outcomes and patient satisfaction and advancing the biomedical and clinical applications of T-hASCs.

Compliance with ethics requirements

All human participants gave their informed consent for the scientific use of their samples, and all procedures involving human samples were approved by the Institutional Review Board of Chung-Ang University Hospital and adhered to the principles outlined in the Declaration of Helsinki guidelines (No. 2151–005–463).

All animal experiments and methodologies employed in this research were approved by the Institutional Animal Care and Use Committees of Chung-Ang University Hospital Institutional Review Board (Approval No. 2021-00030 and 2024-01030046).

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CRediT authorship contribution statement

Chanutchamon Sutthiwanjampa: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Seung Hyun Kang:** Investigation, Methodology. **Mi Kyung Kim:** Investigation, Data curation. **Jin Hwa Choi:** Resources, Investigation. **Han Koo Kim:** Resources, Project administration. **Soo Hyun Woo:** Resources, Project administration. **Tae Hui Bae:** Resources, Project administration. **Woo Joo Kim:** Methodology, Visualization. **Shin Hyuk Kang:** Resources, Conceptualization, Methodology, Project administration, Funding acquisition, Supervision. **Hansoo Park:** Resources, Conceptualization, Methodology, Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2024.07.011>.

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