



Silk fibroin scaffolds potentiate immunomodulatory function of human mesenchymal stromal cells

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ABSTRACT

Although mesenchymal stromal cells (MSCs) show great potential for use in regenerative medicine, their therapeutic efficacy remains limited because of their low adaptation efficiency and viability observed in clinical trials.

To potentiate the adaptation and survival efficiency of MSCs after administration *in vivo*, silk fibroin nanofibers (SFNs) were applied as a scaffold. SFNs are biocompatible, biodegradable polymers with tunable architectures and mechanical properties. Treatment with interferon (IFN)- γ for 18 h increased the expression of immunomodulatory functional cytokines, IDO and COX2 in MSCs. Further, the MSCs grown on SFN sheets showed enhanced IDO1 and COX2 expression following IFN- γ treatment. MSCs showed significantly greater migratory ability on SFN sheets than on glass surfaces or PLGA control sheets. Though IFN- γ treatment slightly reduced the migration ability of MSCs cultured on glass or poly(lactic-co-glycolic acid) (PLGA) nanofiber sheets, it did not alter MSC motility on SFN sheets. Furthermore, MSCs cultured on SFN sheets dramatically suppressed TNF- α secretion from lipopolysaccharide-activated murine splenocytes, suggesting that the immunomodulatory function of MSCs was enhanced by the SFN sheets. Taken together, these data demonstrate that SFN sheets potentiate the reparative and regenerative properties of MSCs.

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1. Introduction

Mesenchymal stromal cells (MSCs) can modulate immunosuppression [1], and their use in clinical practice has increased considerably over the last decade [2]. MSCs limit the innate immune response by inhibiting the maturation and antigen-presenting capacity of dendritic cells [3–5] and decreasing the proliferation and cytotoxicity of natural killer cells [6,7]. MSCs also suppress the adaptive immune response by dampening CD4⁺ helper and CD8⁺ cytotoxic T cell proliferation and functions [8,9]. Moreover, because MSCs can modulate the immune response, they have been used to treat diseases such as graft-versus-host disease, inflammatory bowel disease, and autoimmune disorders [10]. However, clinical trials revealed the poor survival of MSCs after

administration *in vivo* [11,12]. Because the immunomodulatory ability of MSCs is highly dependent on their adaptation efficiency and survival rate after *in vivo* administration, several strategies for improving MSC functions have been explored. In particular, various biomaterials including hydrogels, ceramic, and metals have been utilized for scaffolds improving immunomodulatory function of MSCs [13].

Silk fibroin from *Bombyx mori* can be used to fabricate biomedical composite materials in several forms including fibers [14], films [14,15], sponges, and hydrogels [16] by virtue of their advantageous mechanical properties, degradability, and biocompatibility. Recently, silk fibroin was utilized as a delivery tool or scaffold material for enhancing MSC applications, e.g., silk fibroin-coated alginate beads improved cell attachment and arrangement as well as MSC delivery for *in vivo* applications [17]. Here, an electrospun silk fibroin nanofiber (SFN) sheet was employed to enhance the immunomodulatory function of MSCs. The electrospun structure of the SFN sheet can mimic the structure of the natural extracellular matrix (ECM), and the scaffold structure varies over time as the polymer nanofibers degrade, allowing seeded cells

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to proliferate and produce their own ECM [18]. Therefore, we hypothesized that SFN sheets could provide a beneficial environment for MSCs to enhance their adaptation efficiency and functional properties.

The proinflammatory cytokine, interferon (IFN)- γ , alone or in combination with tumor necrosis factor- α (TNF- α), interleukin (IL)-1 α , or IL-1 β induces MSCs to secrete factors such as cyclooxygenase 2 (COX2), prostaglandin E2 (PGE₂), and indoleamine 2,3-dioxygenase (IDO) [19–21]. In the present study, we evaluated whether SFN sheets could improve the immunomodulatory function and immunosuppressive properties of MSCs by IFN- γ .

2. Materials and methods

2.1. Scaffold fabrication and characterization

Silk fibroin (SF) was obtained from the cocoons of *B. mori* silkworm (Boeun silkworm farm, Chungbook, South Korea). Detail method for preparing silk solution can be found in supplementary information. Electrospinning of the prepared SF solution was performed by placing the solution in 10-mL syringes with a 21-G needle. A syringe pump at a rate of 0.05 mL/h was used to supply the solution to the needle tip. Electric potential (23 kV) was applied between the needle tip (anode) and collecting plate (cathode) at a distance of 17 cm. Electrospun SFN scaffolds were formed on the surface of a glass coverslip (diameter, 25 mm) on the collecting plate by evaporating the solution and via the effect of an electric field. The chemical properties of SF powder were analyzed by Fourier transform infrared spectroscopy (IFS-66/S, Bruker, Billerica, MA, USA). SFN morphology was imaged using a field emission scanning electron microscope (JSM 890, JEOL Ltd., Tokyo, Japan).

To determine the molecular weight of the silk fibroin protein, the protein was boiled in sample buffer (Bio-Rad, Hercules, CA, USA) [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% lithium dodecyl sulfate (LDS), and 0.005% bromophenol blue] and separated by a 12% SDS-PAGE. Protein bands were visualized by staining with 0.05% Coomassie brilliant blue R-250 (Biosesang, Gyeonggi-do, Korea). Briefly, after electrophoresis, the gel was fixed (50% methanol, 10% glacial acetic acid) for 1 h, and then stained with 0.05% Coomassie brilliant blue R-250 (50% methanol, 0.05% Coomassie brilliant blue R-250, 10% acetic acid) for 20 min, followed by destaining (40% methanol, 10% glacial acetic acid).

2.2. Cell culture and ultrastructural analysis

Human bone marrow MSCs were derived from whole bone marrow from independent human donors (commercially available by AllCells, Alameda, CA, USA). Briefly, the cells were cultured in Modified Eagle Medium- α (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (Thermo Fisher Scientific), and 1% antibiotic-antimycotic (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO₂ at 37 °C. MSCs were cultured on SFN or poly(lactic-co-glycolic acid) (PLGA) nanofiber sheets until ~90% confluence, and treated with 100 ng/mL human IFN- γ (Peprotech, Rocky Hill, NJ, USA). All experiments were performed using MSCs from passages 4 to 6.

2.3. Proliferation assay

A cell viability assay kit (MediFab, Rolleston, New Zealand) was used to evaluate the effect of the SFN or PLGA sheet on cell proliferation. MSCs were cultured on a glass coverslip or SFN or PLGA nanofiber sheet-coated glass coverslip attached to a 6-well plate until ~90% confluence and treated with 100 ng/mL IFN- γ for 24 h. After treatment, the viability assay kit solution (1/10 volume of the

culture medium) was added to each well followed by additional incubation for 2 h at 37 °C. Each well was read at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Live imaging and motility analysis

To quantify cell motility, a glass coverslip or SFN or PLGA nanofiber sheet-coated glass coverslip attached to a 6-well plate was placed under an inverted microscope (Nikon Ti-2, Tokyo, Japan) and cellular motility was observed. Successive images were recorded every 10 min for 18 h in an environmental chamber maintained at 37 °C, and containing 5% CO₂. Cells were tracked in time-lapse image sequences using the manual tracking plug-in for ImageJ 1.46r (<http://rsb.info.nih.gov/ij>; NIH, Bethesda, MD, USA). The ImageJ output was integrated into the Chemotaxis and Migration tool software (ibidi GmbH, Gräfelfing, Germany) to determine the migration position and velocity of cells in each well. Migration data for each cell in one position were averaged over the entire cell population (>20 cells for each position, 3 positions per device, and two or more devices for each condition).

2.5. RNA extraction and quantitative real-time PCR

Total RNA was extracted from 1×10^6 cells with the QIAGEN RNeasy Kit (QIAGEN, Hilden, Germany). Reverse transcription of RNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and SYBR Green Real Time PCR (Roche, Basel, Switzerland) was performed in 20- μ L reactions on a LightCycler 96 (Roche). Primer information can be found in supplementary information.

2.6. Immunoblotting

Cells were harvested in RIPA buffer (Thermo Fisher Scientific) containing 1% protease inhibitor cocktail (GenDEPOT, Katy, TX, USA) and 1% phosphatase inhibitor cocktail (GenDEPOT). Equal amounts of protein were separated by SDS-PAGE on Novex 4–20% Tris-Glycine Mini Protein Gels (1.0 mm, 10 well) and analyzed by immunoblotting. Western blotting was performed using standard procedures with rabbit anti-COX2 (Abcam, Cambridge, UK, ab102005), mouse anti-IDO 2,3-dioxygenase (Abcam, ab55305), and beta-actin (Santa Cruz Biotechnology, Dallas, TX, sc-47778). Gel images were captured using SuperSignal West Femto (Thermo Fisher Scientific) in LAS 4000 (GE Healthxare Life Sciences, USA) and the density of the protein bands was quantified using ImageJ software.

2.7. Measurement of IL-10 and TNF- α production

Human bone marrow-derived MSCs were cultured at a density of 2×10^5 cells/mL. Murine splenocytes were isolated from 5-week-old ICR mice for co-culture at 6×10^6 cells/mL with MSCs. Briefly, the spleen was mechanically dissociated through a 70- μ m strainer, lysed in Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich), and resuspended in medium (MEM- α , 20% FBS, 1% AA) after washing with PBS for culture with MSCs. Splenocyte-MSC co-cultures were plated at a ratio of 30 to 1. Lipopolysaccharide (LPS; 1 μ g/mL; Sigma Aldrich) was added 30 min after plating. Following 3 h incubation at 37 °C, the supernatant was collected and analyzed for TNF- α using the Cymax™ Mouse TNF α ELISA kit (Young in Frontier, Seoul, Korea) and IL-10 using the Mouse IL-10 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

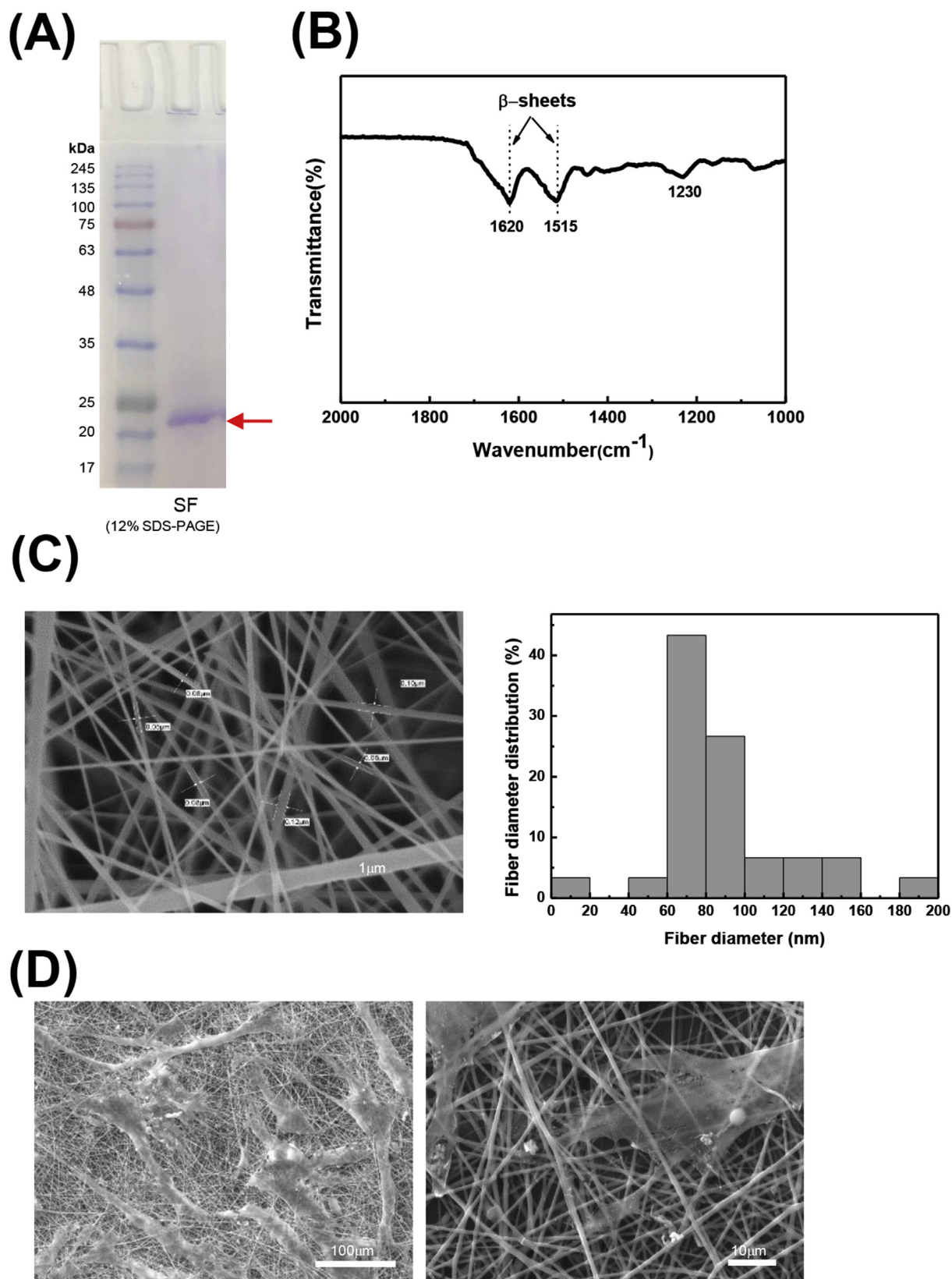


Fig. 1. Properties and characterization of silk fibroin nanofiber sheet. (A) Molecular weight of silk fibroin peptide as determined by SDS-PAGE. (B) FT-IR spectra of SF solids (C) Scanning electron microscopy (SEM) images of the SFNs and histograms showing the diameter distribution of SFNs. (D) MSCs on SFN sheet fixed by 2% glutaraldehyde and ultrastructural images obtained by SEM. Scale bar = 100 μm and 10 μm in each image.

2.8. Statistical analysis

All data were analyzed with GraphPad Prism 8 software (GraphPad, Inc., La Jolla, CA, USA) to determine statistical significance and are reported as the mean $\pm$ S.E.M. One-way analysis of variance (ANOVA) and Tukey's method for multiple comparisons were used to evaluate differences in motility and gene expression across three or more treatment groups.

3. Results

3.1. Fabrication and characterization of SFN sheet

Before the SFN sheet was generated by electrospinning, the molecular weight of SF protein was evaluated by SDS-PAGE. As shown in Fig. 1A, a band for silk fibroin corresponding to a molecular weight of 23 kDa was observed. The FTIR spectra of SF solids showed vibration bands near 1620, 1515, and 1230 cm^{-1} as specific peaks for the SF group in Fig. 1B. The vibration band at 1620 cm^{-1} indicates amide I (C–N bonding), the band at 1515 cm^{-1} indicates amide II (secondary NH bending), and the band at 1230 cm^{-1} indicates amide III (C–C–N bending). The two strong bands of 1515 and 1620 cm^{-1} indicate β -sheet formation in the secondary structure of SF as reported previously [22–24]. The electrospun morphology of SFNs exhibited a uniform distribution and average nanofiber diameters of 82.33 ± 33.90 nm ($N=30$); the diameter distribution of the fibers is shown in the histogram in Fig. 1C.

After generating a sheet structure using SF, human bone marrow-derived MSCs were seeded onto the sheet and ultrastructural analysis was performed by scanning electron microscope (SEM). MSCs were well attached and integrated with SFNs (Fig. 1D).

3.2. Potentiated expression of immunomodulatory cytokines in MSCs cultured on SFN by IFN- γ

IFN- γ regulates the immunomodulatory function of MSCs by inducing IDO expression; thus, studies have evaluated IFN- γ treatment in MSCs to elevate their immune-related functions [25–27]. We investigated whether the 3D structure of the SFN sheets improved the IFN- γ -treated immunomodulatory function of MSCs. Here, MSCs were cultured on either SFN sheets, glass coverslips, or PLGA nanofiber sheets. The PLGA nanofiber sheets showed a 3D structure similar to that of SFN sheets. MSCs were incubated with IFN- γ for 18 h and then COX2 and IDO mRNA expression were evaluated. We previously reported that MSCs mediated immunomodulatory function through the COX2-PGE₂ axis [28]. IFN- γ treatment significantly increased COX2 and IDO transcript levels in MSCs grown on all surfaces (Fig. 2A). Interestingly, MSCs cultured on SFN sheets showed greater enhancement in COX2 and IDO mRNA levels. IDO protein expression levels were also dramatically elevated in MSCs cultured on the SFN sheet with IFN- γ (Fig. 2B), indicating that the 3D structure of SFN sheets enhances the release of immune modulation-related cytokines from MSCs.

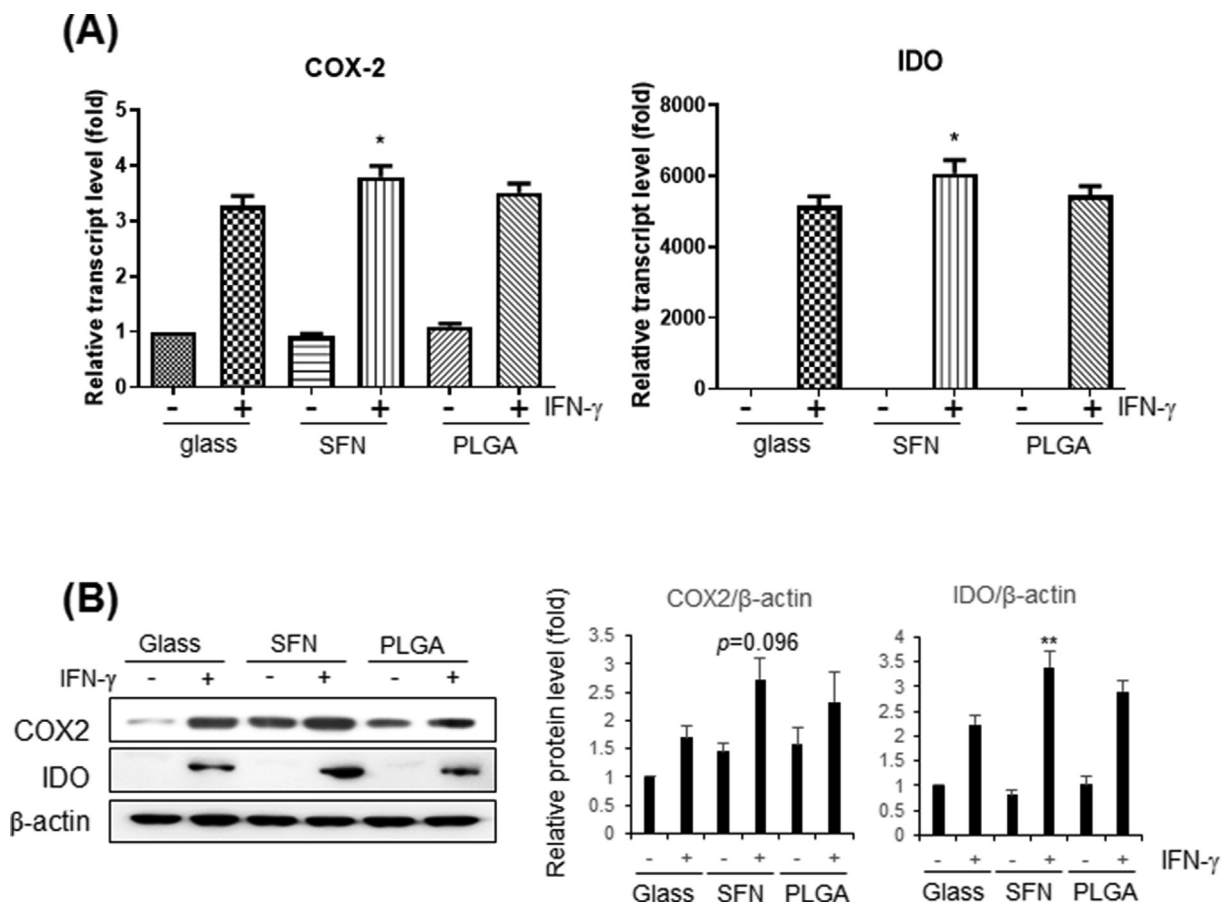


Fig. 2. Potentiated expression of COX2 and IDO in MSCs grown on SFN. (A) COX2 and IDO transcript levels in MSCs are increased upon IFN- γ treatment. SFN significantly enhanced COX2 and IDO mRNA expression in IFN- γ -treated MSCs. Results are expressed as the mean $\pm$ SEM (* $P < 0.05$ relative to glass with IFN- γ ; one-way ANOVA). (B) Treatment with IFN- γ for 18 h further increases COX2 and IDO protein expression in SFN group compared to control surface group (** $P < 0.001$ relative to glass with IFN- γ ; one-way ANOVA).

3.3. Enhanced motility and migration velocity of MSCs cultured on SFN

Although IFN- γ increases the immunomodulatory function of MSCs by inducing COX2 and IDO expression, MSC proliferation rate was reduced [29]. Here we investigated whether SFN sheets attenuate the IFN- γ -induced reduction in MSC proliferation rate. Cells were seeded into a 96-well plate and treated with IFN- γ . A mitochondria activity-based viability assay was performed. MSCs on each surface showed a similar proliferation rate without IFN- γ

(Fig. 3A). Following IFN- γ treatment, the proliferation rate of MSCs was significantly reduced regardless of the cell culture surface, which is consistent with a previous result [29]. Our results indicate SFN sheet did not alter the reduced proliferation rate of MSCs by IFN- γ . Because the homing or migration ability of MSCs appears to be correlated with their therapeutic efficiency [30], the effect of IFN- γ on MSC motility was evaluated. We monitored the motility by time-lapse imaging of cultures on each surface. IFN- γ decreased MSC migration on glass or PLGA sheets, while it did not decrease the motility of MSCs grown on SFN sheets (Fig. 3B and C). Overall,

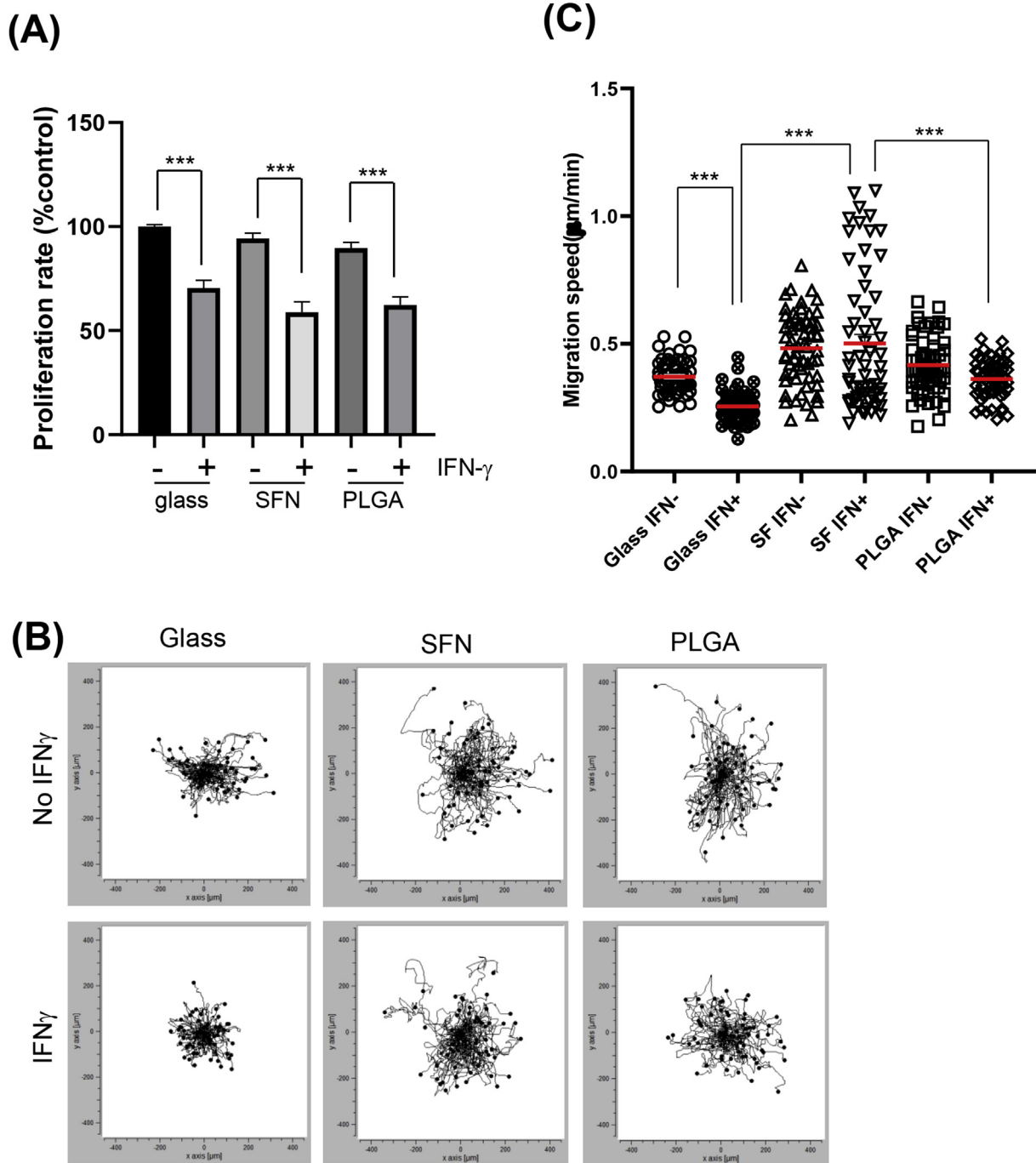


Fig. 3. SFN enhances the migration ability of MSCs. (A) Treatment with IFN- γ for 18 h reduces the proliferation rate of MSCs on glass, SFN sheet or PLGA sheet ($***P < 0.0001$ relative to without IFN- γ ; one-way ANOVA). (B) Migration path of individual cells shows that IFN- γ slows down the motility of MSCs on glass or PLGA sheet. MSC motility on SFN sheet was not altered by IFN- γ . (C) Migration speed of individual cells was quantified (one-way ANOVA; $***P < 0.0001$). Results are expressed as the mean $\pm$ SEM.

MSCs cultured on SFN sheets showed the highest migration velocity compared to those grown on other surfaces, indicating that SFN sheets can improve therapeutic efficiency.

3.4. Effective suppression of TNF- α production by MSCs cultured on SFN

Here, we found that the SFN sheet enhanced COX2 and IDO expression in MSCs. To evaluate the anti-inflammatory function of MSCs, we established co-cultures of MSCs with murine immune cells isolated from the spleen, consisting of macrophages, neutrophils, NK, B, and T cells. IFN- γ was incubated with MSCs on each surface followed by co-culture with LPS-treated murine splenocytes. In response to LPS, murine splenocytes produce pro- and anti-inflammatory cytokines including IL-10 and TNF- α . MSCs cultured on SFN sheets enhanced the production of IL-10 and suppression of TNF- α secretion by the splenocytes (Fig. 4A and B). Thus, SFN sheets enhanced the migration ability and immunomodulatory function of MSCs, suggesting that MSCs grown on SFN sheets can enhance therapeutic potency.

4. Discussion

We demonstrated that the immunomodulatory function of MSCs can be enhanced by culturing on SFN sheets. These sheets showed increased COX2 and IDO expression in IFN- γ -stimulated MSCs compared to those grown on the control surface or PLGA sheet. Furthermore, SFN sheets attenuated the IFN- γ treatment-induced reduced motility of MSCs, whereas MSCs grown on the control surface or PLGA sheet were slowed by IFN- γ . Enhanced COX2 and IDO expression in MSCs cultured on the SFN sheet was correlated with their increased TNF- α suppression capacity, suggesting that SFN sheets potentiate the immunomodulatory function of MSCs.

MSCs are beneficial for treating many immune-associated diseases. However, long-term survival and adaptation of MSCs after transplantation remain limited [31]. Transplanted MSCs exhibit cell death within a few days after transplantation because of their inability to adapt to the harsh microenvironment in the body [32]. Therefore, these properties of MSCs must be improved before *in vivo* application; towards this, several approaches have been explored, such as bioactive factors, preconditioning, genetic modification, and bioengineered molecules [33,34].

Here, electrospun SF was utilized as a culture surface because it increases cell adhesion and proliferation, and has good biocompatibility, oxygen and water vapor permeability, biodegradability, and low inflammatory responses [35]. Moreover, SFN sheets generate 3D structures that mimic the natural ECM. Signaling from matrix-derived cues affects a cellular response known as mechanotransduction. Through this process, cells indicate outside-in signals and alter gene/protein expression. These expression profiles can further alter the extracellular environment through an inside-out signaling mechanism [36,37]. In this study, 3D structure surfaces such as SFN or PLGA sheets showed enhanced TNF- α suppression by MSCs, although the PLGA sheets did not increase COX2 and IDO expression (Figs. 2 and 4), indicating that the biomimetic structure improved the immunomodulatory potential of MSCs. Similarly, Darzi et al. showed that MSCs grown on a synthetic composite mesh significantly reduced the inflammatory response in immunocompromised mice, while injected MSCs without a scaffold did not survive at the site [38]. Thus, a 3D-structured scaffold with SFN generates a beneficial environment for MSCs and improves immunomodulatory function.

COX2 and IDO levels were evaluated as markers of immunomodulation in IFN- γ -stimulated MSCs. Interestingly, SFN sheets significantly increased COX2 and IDO expression at the mRNA and protein levels after IFN- γ treatment, whereas PLGA sheets showed a low induction ability compared to the glass surface. COX2 is the rate-limiting enzyme in PGE₂ metabolism. PGE₂ signaling mediates the promotion of anti-inflammatory phenotypes in macrophages and inhibition of NK cell cytotoxicity [39]. In our previous study, the immune regulatory function of MSCs was found to be mediated via the NF- κ B-COX2-PGE₂ axis [28]. Furthermore, IDO expression is necessary for MSC-mediated suppression of T-cell proliferation, and variable amounts of IDO expression between MSCs donors are strongly correlated with the ability to suppress T-cell responses [19,40]. In addition to directly modulating the T cells, MSCs induce IL-10-expressing monocytes within PBMC co-cultures to aid T-cell suppression *in vitro* [40]. Therefore, increased IL-10 expression from splenocytes by the action of MSCs cultured on SFN sheets may increase the immunosuppressive activity of MSCs (Fig. 4). In summary, our data suggest that SFN sheets are an ECM-mimicking scaffold that provide an effective platform for enhancing MSC immunomodulatory activity for cellular therapies. Further studies will be required to precisely show improved immunomodulatory function of MSCs *in vivo* model.

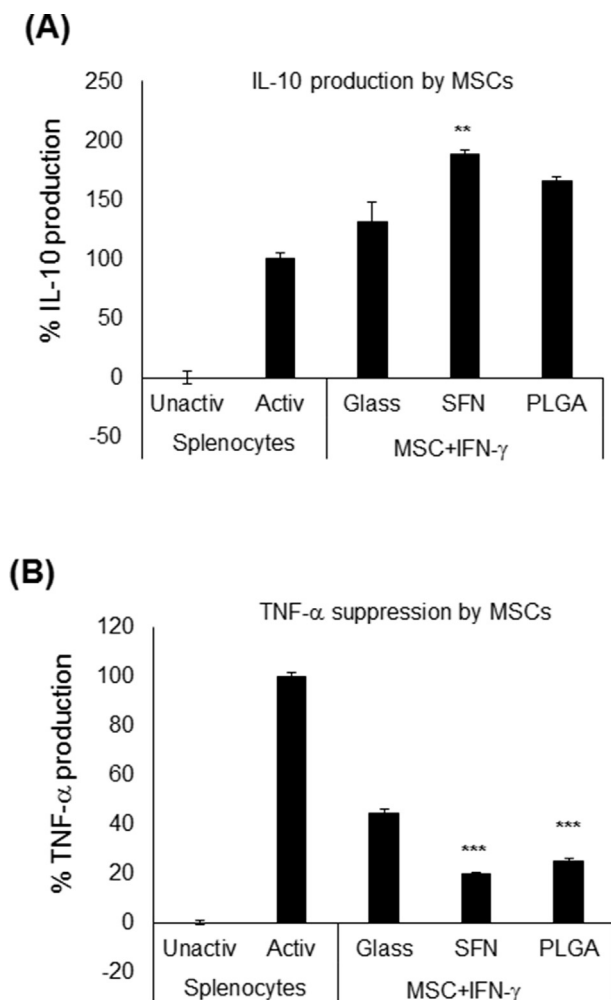


Fig. 4. Immunomodulatory function of MSCs are amplified by SFN scaffold. (A) IL-10 is elevated by SFN sheet (** P < 0.001, SFN vs other groups; one-way ANOVA). (B) MSCs grown on SFN sheet suppress the secretion of TNF- α from murine splenocytes more potently (one-way ANOVA; *** P < 0.0001 relative to glass).

Disclosure

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.09.006>.

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